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Influence of Excessive Amounts of Vit. D_3 on Strontium-89 Metabolism in the Rat.* (25705)

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With the increase in use of nuclear power there is also concomitant jeopardy from fission products. Contamination of areas particularly with strontium-89 and -90 through explosions of nuclear devices and through wastes from nuclear reactors is coming into the limelight as an international problem. Hamilton(1) reported that these isotopes are available from food which might be produced on contaminated land. The primary site of deposition of strontium nuclides in the body is in the skeleton, where they are retained for long period emitting beta particles which may seriously impair the blood forming functions of bone marrow and induce bone tumors(2). Investigators have reported a clearcut reduction of strontium-90 burden with increased ingestion of calcium(3,4). Pugsley(5) observed a decrease in fecal calcium with an increase in urinary calcium during administration of irradiated ergosterol. Patrick and Bacon(6) have shown that use of Vit. D increases the efficiency of utilization of Ca^{45} and Sr^{89} in both organic and inorganic forms in the rat. This study was initiated to ascertain the effect of feeding excessive amounts of Vit. D_3 in presence and absence of dietary calcium or strontium on strontium-89 metabolism in the rat.

Methods and materials. A total of 116 weanling albino rats (Sprague-Dawley strain) were used with a minimum of 4 rats per diet in each of the experiments. The diet reported

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TABLE I. Strontium-89 Content of Excreta of Rats Fed Various Levels of Vit. D_3.

I.U. D_3 added to 1 kg basal diet (.5% Ca)	Excretion (%)*		
	Fecal	Urinary	Total
None	53.6	4.9	58.5
7,500	53.1	6.1	59.2
75,000	37.7	10.8	48.5
100,000	35.6	8.0	43.6
200,000	20.2	20.5	40.7
300,000	25.0	18.9	43.9
400,000	14.3	25.0	39.3
500,000	16.7	28.4	45.1
1,000,000	13.1	32.4	45.5
2,500,000	11.0	30.9	41.9
L. S. D.†	11.7	7.4	11.0

* Expressed as % of orally administered dose excreted in 72 hr.

† Least significant difference between means at 5% level of confidence.

by Hansard *et al.*(7) was used with slight modifications in the mineral mixture. Adjustments in minerals and Vit. D were made at the expense of cornstarch. Ten microcuries of strontium-89 in the chloride form were orally administered to each rat. They were then placed in metabolism units described by Hansard and Comar(8) and excreta collected daily. All samples were ashed at 600°C, dissolved in hydrochloric acid, precipitated with ammonium oxalate and counted with an end-window Geiger-Muller tube with a window thickness of 1.4 mg/cm^2. In the first experiment a Vit. D deficient diet (containing 0.5% calcium) was supplemented with levels of Vit. D_3 shown in Table I to ascertain the level necessary to produce a change in strontium-89 excretion. These diets were fed for a period of 7 days before administration of the radionuclide after which time a 72-hour balance trial was conducted. The influence of dietary calcium and strontium on excretory strontium-89 response to excessive Vit. D_3 feeding was determined in the second experiment (Table II). A calcium deficient diet was supplemented was 0.75% calcium and/or an equivalent amount of strontium (1.64%). Each of these 4 diets had a counterpart with 200,000 I.U. of Vit. D_3/kg. Rats were fed these diets for a period of 6 days before administration of strontium-89 at which time a 72-hour balance trial was initiated. In the third experiment the influence of length of period of feeding Vit. D_3 and dihydrotachysterol (a reduction product of tachysterol which is a product of irradiation of ergosterol promoting phosphaturia and mobilizing calcium from the skeleton(9)) on strontium metabolism was ascertained (Table III). Two diets were formulated from the basal diet (containing 0.5% calcium) which was deficient in Vit. D; one contained 2,500,000 I.U./kg Vit. D_3 and the other contained 2.5 mg/kg dihydrotachysterol. Three groups of rats were started on the basal diet 3 days before administration of the radionuclide and the other 2 were fed either the Vit. D_3 or dihydrotachysterol diet.

TABLE II. Strontium-89 Content of Excreta of Rats Fed Varying Levels of Strontium, Calcium and Vit. D_3.

Supplements to Ca-deficient basal diet	Excretion (%)*		
	Fecal	Urinary	Total
None	23.5	4.2	27.7
+ .75% Ca	60.5	3.0	63.6
+ 1.64% Sr	45.5	12.2	57.7
+.75% Ca + 1.64% Sr	64.0	13.3	77.3
+200,000 I.U. vit. D_3/kg	28.7	4.4	33.1
+ .75% Ca + 200,000 I.U. vit. D_3/kg	31.6	14.5	46.1
+ 1.64% Sr + 200,000 I.U. vit. D_3/kg	21.9	24.1	46.0
+ .75% Ca + 1.64% Sr + 200,000 I.U. vit. D_3/kg	32.8	28.7	61.5
L. S. D.†	10.2	7.4	10.0

* Expressed as % of orally administered dose excreted in 72 hr.

† Least significant difference between means at 5% level of confidence.

TABLE III. Strontium-89 Content of Excreta of Rats Fed Vit. D_3 or Dihydrotachysterol 3 Days Before or at Time of Administration of Sr^{89}.

Supplements to basal diet (.5% Ca)	Days fed before Sr^{89} admin.	Excretion (%)*		
		Fecal	Urinary	Total
None	3	28.7	3.9	32.6
Vit. D_3 (2,500,000 I.U./kg)	3	11.3	30.4	41.7
Idem	0	35.3	10.4	45.7
Dihydrotachysterol (2.5 mg/kg)	3	28.1	10.7	38.8
Idem	0	28.9	5.6	34.5
L. S. D.†		5.4	1.5	5.4

* Expressed as % of orally administered dose excreted in 96 hr.

† Least significant difference between means at 5% level of confidence.

At time of administration of strontium-89, these latter diets were fed to 2 of the groups started on the basal diet. To allow more time for the effect of the diets to be shown, a 96-hour balance trial was made.

Results. Fecal excretion of strontium-89 tended to be less in diets containing more Vit. D_3 with significantly smaller amounts occurring between the 7,500 and 75,000 I.U. levels and the 100,000 and 200,000 I.U. levels of Vit. D_3 supplementation (Table I). The converse was true for urinary excretion of strontium-89 with significant increases first being shown between the 100,000 and 200,000 I.U. levels of Vit. D_3 supplementation. The increase in urinary strontium-89 was not sufficient to compensate for the decrease in fecal strontium-89 and therefore, significant decreases in total excretion of strontium-89 were apparent. While total excretion of strontium-89 was not greatly affected by massive Vit. D_3 supplementation, the amount passing through the gutwall was increased almost 5-fold as measured by fecal strontium-89. It would appear, therefore, that Vit. D_3 has the property of decreasing fecal excretion.

In the absence of dietary calcium or strontium, no significant effect on strontium-89 excretion was observed from feeding 200,000 I.U. Vit. D_3/kg of diet (Table II). However, in the diets containing calcium or strontium, the significant decreases in fecal strontium-89 and increases in urinary strontium-89 observed in the previous experiment were again shown. It would also appear that high levels of Vit. D_3 add to the effect of dietary strontium in increasing urinary and decreasing fecal strontium-89.

In every instance (Table III) inclusion of either Vit. D_3 or dihydrotachysterol significantly increased urinary excretion of strontium-89. However, the 3-day feeding period before radionuclide administration was significantly more effective in producing this effect. The level of Vit. D_3 used (2,500,000 I.U./kg) was considerably more efficient in increasing urinary strontium-89 than was the level of dihydrotachysterol used (2.5 mg/kg). While Vit. D_3 influenced fecal strontium-89, dihydrotachysterol exerted no such effect. Over these short periods, high levels of Vit. D_3 significantly increased total excretion of strontium-89.

It appears from our data that concentrations of calcium or strontium are necessary in the diet to exhibit the effect of excessive amounts of dietary Vit. D_3 on excretory pattern of strontium-89. A preliminary period of Vit. D_3 feeding before dosing was required for maximum expression of this effect. As would be expected from its effect on calcium metabolism(9), dihydrotachysterol did not appreciably influence intestinal absorption of strontium-89, but did increase its urinary excretion probably through mobilization from the skeletal pool. The increased urinary excretion of strontium-89 following Vit. D_3 feeding, however, is a reflection of its mobilization from the skeleton as well as of the increased intestinal absorption of strontium-89.

Summary. The experiments demonstrated that Vit. D_3 exerted an effect on excretion of strontium-89 by increasing absorption of strontium-89 from the gut, by decreasing fecal and increasing urinary strontium-89 providing either calcium or strontium is present in the diet. The efficiency with which Vit. D_3 exerted this effect was governed by its concentration in the diet and by length of time it was fed before administration of strontium-89.

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Insulin-Like Activity of Crystalline Glucagon as Measured with Rat Epididymal Fat Pad Preparation. (25706)

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Crystalline preparations of glucagon were reported by Staub *et al.*(1) to contain approximately 0.05 unit/mg insulin activity. This was measured by the mouse convulsion assay. Review of literature on glucagon by Behrens and Bromer(2) revealed conflicting data on insulin-like activity of glucagon. Some workers found high concentrations of glucagon inhibited response of insulin. Others reported low concentrations of hormone augmented uptake of glucose by muscle, an insulin-like effect. Two reports, relating to insulin content of both amorphous and crystalline glucagon, have appeared recently. McNaught(3) used slices of lactating rat mammary gland to study effect of crystalline glucagon, lot No. 258-234B-43, prepared in this laboratory. Responses measured were increase in gas output and augmented uptake of glucose. The order of insulin-like activity for this sample, estimated by both responses, was 1.08%. Randle(4) likewise reported that measurable amounts of insulin-like activity were found in 3 lots of glucagon tested by isolated rat diaphragm method. The amorphous sample contained equivalent of 1.2% insulin, whereas remaining lots, including crystalline, lot No. 258-234B-54-2, were far less active. Both McNaught and Randle found that pretreatment of glucagon with cysteine completely eliminated the insulin-like response. During a study of rat epididymal fat pad preparation of Martin *et al.*(5), our preliminary tests indicated that crystalline glucagon increased oxidation of glucose in the same fashion as insulin. This was shown by its augmentation of $C^{14}O_2$ output when glucose-1-C^{14} was the labeled substrate. Quantitative data comparing the effects of crystalline glucagon, crystalline glucagon (cysteine-treated), and insulin, using a modification of the Martin method as reported here. Observations on the action of these hormones on glucose uptake of the rat epididymal fat pad are also included.

Material and methods. Crystalline glucagon lot Nos. 258-234B-43 and 258-234B-54-2, reported by McNaught and Randle, respectively, and lot No. 258-234B-167-1 were compared with insulin. Lot No. 258-234B-167-1 was tested both before and after treatment with cysteine. Inactivation of insulin content of glucagon with cysteine consisted of incubation of a 1% solution of crystalline glucagon with 1% cysteine at pH 8.7 overnight at 10-15°C. This was followed by dialysis against water, lyophilization, and recrystallization of glucagon from a pH 9 water solution at 4°C. Approximately 50% glucagon was recovered. No glucagon activity was lost after cysteine treatment. *Crystalline insulin* (25.3 units/mg), trypsin-treated and low in glucagon, was used as reference standard. Separate fresh stock solutions of insulin and glucagon were made up daily using Krebs bicarbonate buffer. N HCl was added to insulin solutions and N NaOH added to glucagon solutions to dissolve the hormones. Care was taken to maintain pH of stock solutions of glucagon at pH 9 or less. The fresh stock solutions were diluted to desired concentrations with Krebs buffer at pH 7.6. Final concentration of glucose in all incubations was 2 mg/ml. To minimize the loss of insulin on glass surfaces, a concentration of 100 μg/ml of crystalline egg albumin was maintained in each dilution made from hormone stock solutions. Tissue incubations with labeled glucose-1-C^{14}, 0.4 μC/flask and varying amounts of hormone were made in 15 ml Warburg flasks in a Dubnoff metabolic shaker at 37°C for 4 hours. Volume of incubation medium was 2 ml. The flasks were gassed 5 minutes initially with a 95% O_2 plus 5% CO_2 gas mixture. The mode of trapping $C^{14}O_2$ in Hyamine solution was essentially the same as that described by Siperstein and Fagan(6). Radioactivity in the Hyamine solution was counted to a precision of 5% or greater in a toluene scintillation mixture containing 0.05%

2,5 diphenyloxazole and 0.001% 1,4-bis-2(5 phenyloxazole) benzene. The counter employed was a liquid scintillation spectrometer (Packard 314X). Addition of an internal standard was added to correct for variation in quenching from sample to sample. The efficiency of transfer of $C^{14}O_2$ into the Hyamine solution was determined experimentally to be of the order of 94%. Radioactivity found as $C^{14}O_2$ was expressed as counts/min/g of tissue wet weight. An initial study of the $C^{14}O_2$ response of tissue to insulin indicated that within the range of 1 to 1,000 μunits/ml, the response varied essentially linearly with the log of concentrations of insulin. Similarly, with crystalline glucagon, a usable range was found between 10 and 100 μg/ml. For quantitative work, a 6 x 6 assay design was used to compare insulin, glucagon, and cysteine-treated glucagon at each of 2 concentrations. Each rat contributed 6 pieces of tissue, thus there were 36 pieces in all (Table I). With glucose uptake experiments, which were made separately, the procedure was altered as follows: volume of incubation medium was 0.8 ml and incubations were made in 10 ml beakers under an atmosphere of 95% O_2 plus 5% CO_2, which was maintained throughout the 4 hours. Glucose concentrations at the end of incubation time were determined in the remaining fluid with an AutoAnalyzer (Technicon), and uptake of glucose was expressed in terms of mg/g wet weight of tissue/4 hours. Male albino rats, non-fasted, weighing 230 to 300 g, were used in both studies. The animals were sacrificed by decapitation with a small guillotine. The epididymal fat tissue was removed, and each pad was cut into 2 or 3 pieces. Each animal thus furnished 4 or 6 pieces of tissue. Minimal handling of tissue and avoidance of chilling were essential for good responses to the various agents. With glucose uptake experiments, a study of the relationship between concentration of agents and response was not attempted. Only one concentration/sample was used. Each animal furnished 4 pieces of tissue, 3 of which were given separate treatments and one was utilized as a control (Table II).

Results. The data on comparison of insulin and the 2 glucagon samples are found in Table I. An analysis of variance of these data showed that the differences due to animals, samples, and concentrations were all greater than that expected due to chance. The calculated constants relating concentration to response for insulin and glucagon are not significantly different. These were respectively 144.8 and 124.4. A combined slope constant 134.54 ± S.E. 34.46 of the data from both glucagon and insulin was, therefore, used to calculate relative insulin-like activity of glucagon on a weight basis. This calculated value indicated that 1 mg of this sample of glucagon, lot No. 258-234B-167-1, was equivalent to 0.134 μg of insulin. The 95% confidence limits were 0.058 to 0.306 μg of insulin. Therefore, this glucagon sample contains as a best estimate 0.013% of insulin-like potency.

The concentration-response data for cysteine-treated glucagon showed a slope constant of 31.75 ± 26.85. Disregarding this difference in slope constant between glucagon and cysteine-treated glucagon, the best estimate of insulin-like activity in this sample was calculated to be 8.45% of the untreated

TABLE I. Comparison of Insulin, Glucagon, and Cysteine-Treated Glucagon on $C^{14}O_2$ Response of Rat Fat Pad in 6 Rats. Glucose-1-C^{14} was the substrate.

	Treatment					
	I_{10}	I_{100}	G_{10}	G_{100}	GT_{10}	GT_{100}
	93	427	218	514	132	225
	55	191	170	279	114	123
	55	244	125	200	106	127
	44	119	96	149	63	92
	118	169	164	284	174	212
	75	158	153	186	112	113
Means	73.3	218	154.3	278	116.8	148.5

Numerical values represent $\frac{\text{cpm} \times 10^3}{\text{g tissue/4 hr}}$.

I_{10} = insulin, 10 μu/ml; I_{100} = insulin, 100 μu/ml; G_{10} = glucagon, 10 μg/ml, lot No. 258-234B-167-1; G_{100} = glucagon, 100 μg/ml, lot No. 258-234B-167-1; GT_{10} = cysteine-treated glucagon, 10 μg/ml, lot No. 258-234B-177-1; GT_{100} = cysteine-treated glucagon, 100 μg/ml, lot No. 258-234B-177-1.

Mean value of $\frac{\text{cpm} \times 10^3}{\text{g tissues}}$ of untreated 14 control tissues = 37.56 ± S.E. 2.21.

Composition of Krebs bicarbonate buffer: K^+ 5 mM, Ca^{++} 0.99 mM, Mg^{++} 0.53 mM, Na^+ 146 mM, Cl^- 114 mM, HCO_3^- 40 mM.

TABLE II. Effect of Insulin, Glucagon, and Cysteine-treated Glucagon on Glucose Uptake of Rat Epididymal Fat Pad.

Sample	No. of tissues tested	Concentration, μu/ml	Mean glucose uptake ± S.E., mg/g/4 hr	t*	p†
Control	4		1.274 ± .145		
Glucagon 258-234B-167-1	4	100	7.94 ± .557	29.2	<.001
Glucagon, cysteine treated 258-234B-177-1	4	100	7.42 ± 1.04	6.15	<.001
Insulin	4	100	6.38 ± 1.34	4.37	<.01

* = t value of significance treated *vs* control.
† = probability of difference arising by chance.

sample, or approximately 0.001% insulin by wt.

Two additional lots of crystalline glucagon were compared with insulin in a similar manner. The results of these comparisons are: Lot No. 258-234B-54-2 = 0.0074% insulin; 95% confidence limits 0.0021-0.0258%; Lot No. 258-234B-43 = 0.08% insulin; 95% confidence limits 0.0235-0.285%.

The data on the glucose uptake experiments are found in Table II. All samples, including glucagon preparations, caused a significant uptake of glucose at concentrations used.

Discussion. Our results differ only quantitatively from those described by Randle and McNaught. In most instances the insulin-like activity of crystalline glucagon, as determined by rat epididymal fat pad method, were lower than those reported with other tissues. The test of the cysteine-treated glucagon did not produce a reliable estimate of activity. We are unable to state that all insulin has been removed from this sample by cysteine treatment. The fact that the slope of concentration-$C^{14}O_2$ response curve has been altered and that this insulin-inactivated sample still caused a significant uptake of glucose, could be used as arguments for an effect of glucagon itself on fat tissue, although the effect is very small in comparison with insulin. A more direct solution to the problem would involve studies similar to those described by Winegrad and co-workers(7), who found that bovine growth hormone increased glucose oxidation but failed to stimulate fat synthesis.

Summary. A small amount of insulin-like activity has been found in crystalline preparations of glucagon with rat epididymal fat pad preparation. Treatment of glucagon with cysteine lowers this insulin-like activity to a small fraction of initial activity.

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Composition of Lymph Cholesterol Ester Fatty Acids after Feeding of Cholesterol and Oleic Acid.* (25707)

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It has been shown(1) that administration of several different fatty acids will increase degree of absorption of administered cholesterol. However, very little data are available on extent to which administered fatty acid appears in cholesterol ester fatty acids of lymph. Clement and Mead(2) reported that following feeding of cholesterol and C^{14}-oleic acid, administered oleic acid in lymph sterol esters was diluted with endogenous oleic, linoleic and stearic acids. It has been shown(3,4) that free cholesterol, after entering mucosa from the lumen, is mixed with free cholesterol pool prior to its esterification and transfer to lymph. Considerable dilution of fed cholesterol-4-C^{14} with endogenous sterol occurred in its transfer from lumen to lymph. If there occurs an endogenous dilution of administered cholesterol during absorption, it also appears likely that the accompanying administered fatty acid would also be diluted with endogenous fatty acid in the mucosa prior to esterification and transfer to the lymph. The present study was undertaken to determine effect of feeding cholesterol and oleic acid on composition of cholesterol ester fatty acid(s) (CEFA) of lymph. For comparative purposes CEFA composition of fasting lymph was also determined.

Methods and materials. Rats with thoracic lymph fistulas were prepared and given saline to drink(3). Twenty-four hours after operation each animal of one group (6 rats) received, by gastric intubation without anesthesia, 3 ml of aqueous emulsion containing 40 mg cholesterol, 292 mg oleic acid, 279 mg sodium taurocholate, 50 mg blood albumin and 150 mg glucose. Lymph was collected from 0-24 hours following administration of test meal. Lymph was also collected from 0-24 hours in a comparable fasting control group (6 animals). Lipid extracts of individual lymph samples were prepared as described earlier(3,4). Free and total cholesterol of each extract were determined by the method of Sperry and Webb(5). Cholesterol esters were separated from other lipid components by chromatography on silicic acid. The isolated cholesterol esters were interesterified in HCl-methanol and the methyl esters were sublimed according to procedure of Stoffel *et al.*(6). Gas-liquid chromatography was carried out as previously described(7) using a succinate polyester of diethylene glycol as the stationary phase(8).

Results. The data on lymph CEFA of both groups of animals are given in Table I. A wide spectrum of CEFA was found in fasting lymph. The major fatty acids present were palmitic, linoleic and oleic acids; those acids comprised 77.1% of the total CEFA. The presence of arachidonic acid (7.2%) is also of interest.

Following feeding of test meal containing cholesterol and oleic acid considerable changes in the other CEFA besides oleic acid occurred in lymph. The oleic acid fraction increased from 16.1 to 42.3%, but in terms of total amount of fatty acid the increase was 6-fold. There were decreases in percentages of palmitic, linoleic and linolenic acids, but on a weight basis there was a slight increase in palmitic acid, while linoleic and linolenic acids remained the same. There were also increases in absolute amounts of the other fatty acids of the CEFA fraction of lymph, the most notable being a 2-fold increase in arachidonic acid level.

Discussion. These results confirmed and extended earlier observations by Clement and Mead(2). Fasting rat lymph contains a wide spectrum of CEFA and it is of interest that a considerable portion (36%) of total CEFA is present as polyunsaturated fatty acids. Comparison of the CEFA composition of fasting

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TABLE I. Cholesterol Ester Fatty Acid Composition of Lymph of Fasted and Cholesterol-Oleic Acid Fed Rats.*

Fatty acid†		% total fatty acids		mg fatty acid/24 hr lymph sample‡	
Chain length carbons	No. double bonds	Fasting	Cholesterol-oleic acid	Fasting	Cholesterol-oleic acid
6 to 12		.5 ± .2§	1.1 ± .4	.03	.12
14	0	.5 ± .2	1.1 ± .3	.03	.12
14	1	.7 ± .2	.6 ± .3	.04	.07
16	0	34.4 ± 3.1	22.3 ± 8.9	1.79	2.52
16	1	2.7 ± .4	7.5 ± 1.8	.14	.85
18	0	9.0 ± 2.4	7.0 ± 1.3	.47	.79
18	1	16.1 ± 2.8	42.3 ± 5.4	.84	4.77
18	2	26.6 ± 2.6	11.6 ± 3.2	1.38	1.31
18	3	2.1 ± 1.7	.7 ± .7	.11	.08
20	4	7.2 ± 2.6	5.6 ± 2.3	.37	.63
20	5	.2 ± .2	tr	.01	tr
Total fatty acid				5.21	11.26

* Values represent avg of 6 animals; lymph collection period 24 hr.
† Represents major fatty acids found; very small amounts of others were also detected.
‡ The mg of individual CEFA in lymph were calculated from total CEFA content of lymph (derived from lymph cholesterol ester levels) and percentage of individual fatty acids.
§ Stand. dev.

lymph with that of fasting rat serum† indicates that they are markedly different. Rat serum CEFA consist predominantly of polyunsaturated fatty acids (70%) with arachidonic acid comprising 50% of the total.

The data clearly show that, when oleic acid was fed, other CEFA than oleic acid changed in amount and in percentage of total acids. If the fed cholesterol had been simply esterified with the dietary oleic acid then amount of cholesterol oleate appearing in lymph should have been substantially greater than that observed. The data are in agreement with the concept that both exogenous cholesterol and fatty acids are mixed with mucosal pools of these substances prior to esterification and transfer to lymph(9). Under these circumstances the composition of lymph CEFA would be a reflection of the composition of the mucosal fatty acid pool after entrance and mixing of the fed oleic acid in the mucosa.

Summary. Fasting lymph and lymph following feeding of a mixture containing cholesterol and oleic acid were analyzed for their CEFA composition by gas-liquid chromatography. The major fatty acids of fasting lymph were palmitic, linoleic and oleic acids, with polyunsaturated fatty acids comprising 36% of total CEFA. After feeding oleic acid only 42.3% of total CEFA was present as oleic acid. Our results support the concept that CEFA composition is not determined solely by dietary fatty acid, but by the composition of the fatty acid pool in the mucosa from which fatty acids are drawn for esterification of cholesterol.

† Unpublished observations.

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Volume and Turnover of Body Water in *Dipodomys deserti* with Tritiated Water.* (25708)

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The Kangaroo rat is of interest to the physiologist because of its rather unique water and electrolyte metabolism. That these animals can exist in xeric habitats, where cacti and succulent vegetation are not available as water sources, is well documented(1,2). If humidity and temperature are controlled, Kangaroo rats can be maintained in positive water balance when fed only dry seeds(2,3). No specialized water depots are known to exist in desert rodents(2,4). Although glomerular filtration rate, kidney size, and number of nephra are essentially the same as in other rats of similar weight(5,6), it is known that the structure of collecting ducts and papilla renis of Heteromyidae is unique among rodents(5, 7). Ames and Van Dyke(8) found high concentrations of antidiuretic hormone in urine and posterior pituitary of the Kangaroo rat. Howell and Gersh(1) suggested that absorption of water from the bladder may also occur; however, this mechanism could not be verified by other investigators(4). Higher concentrations of electrolytes in urine(9) and greater reabsorption of water from tubuli of Heteromyidae, as compared with the white rat(10), also have been demonstrated. One of the results of preceding mechanisms, which contributes to unique water economy of this animal, is that of a prolonged body water turnover time. However, comparatively little is known about the kinetics of water metabolism in the desert rat. Our purpose was to determine volume and turnover of exchangeable body water (using tritium water dilution method) as adjunct to a study of interspecific relations in water metabolism.

Materials and methods. The animals (*Dipodomys deserti*) were live-trapped in the Nevada desert,† and morphological characters were used to identify the species. Animals were housed individually in small galvanized sheet-metal cages containing dusty Nevada sand and covered with hardware cloth. Animal room was darkened and relative humidity maintained at 20-30% at 70-75°F. During 18-day acclimatization period, the animals were given pearled barley *ad lib.* and only an occasional slice of apple to serve as exogenous source of water. Pearled barley, which offers several advantages as food source, was kept in open container to assure equilibration of preformed water (*i.e.*, that water which can be removed by drying at 105°C) with the water in air. Two experiments were performed to determine turnover of exchangeable body water. The first (Group A) started after initial 18-day acclimatization, and the second (Group B) began about 80 days later following additional 14-day acclimatization in different animal quarters. Temperature in latter quarters ranged between 70-75°F, and relative humidity 10-20%. Each rat was injected intraperitoneally with 0.1 ml water containing 0.85 mc of tritium as HTO. At 2, 3, and 4 hours after injection, 0.02 ml of blood was drawn into a Sahli hemoglobin pipette from small incision in tail vein and mixed with 1 ml of normal saline. After centrifuging, 0.5 ml of clear supernatant was assayed for tritium activity using a liquid scintillation method. Details of assay procedure have been reported by Langham *et al.*(11). Tritium activity of each sample was converted to μc/ml of body water by multiplying by appropriate dilution factors and assuming blood to be 79% water. Equilibration of HTO with body water was determined from 3 blood samples obtained on first day, and turnover was calculated from changes in HTO concentration in 5 blood samples obtained for 18-day period.

Results. The mean exchangeable body water content for 20 animals was 62.37% of body weight, with standard deviation (σ) 2.16. No

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† We acknowledge our appreciation to Dr. Kermit Larsen of Environmental Radiation Division, Univ. of California at Los Angeles, for these animals.

TABLE I. Mean Exchangeable Body Water Content of *Dipodomys deserti*.

Group	No. of animals	Mean body water content (% of body wt)
A	8	62.00 ± 2.17*
B	12	62.63 ± 2.21
Total	20	62.37 ± 2.16

$$^{*}\sigma = \pm\sqrt{\frac{\Sigma x^2 - \Sigma x\,(\bar{x})}{n-1}}.$$

statistically significant difference between mean exchangeable body water content of the 2 groups was observed (Table I), and the turnover of HTO followed a single exponential function throughout experimental period (Fig. 1). The half-time ($T_{½}$) of body water turnover of each group can be determined from the relationship $T_{½} = \frac{0.693}{k}$, where k is the rate constant. The mean life (τ) of HTO in each group is equal to reciprocal of rate constant. Table II lists values of parameters a and k of the exponential rate equations and $T_{½}$ for individual animals. Values for a and k were obtained from regression equations calculated by the method of least squares.

Water intake for animals in Group A was estimated from food consumption and compared with the value calculated from exchangeable body water and turnover data shown in Table II and Fig. 1. Mean exchangeable body water content at time zero is the product of body weight and the fraction of body weight which is water (59.41 g water). As loss is exponential, the amount remaining in the body at end of first day (A_1) can be calculated from the following relationship (where A_0 is equal to 59.41 g, and t is equal to 1 day): $A_1 = A_0e^{-kt} = 55.23$ g water. The difference between A_0 and A_1 is equal to loss during first 24 hours, which amounts to about 4.2 g water.

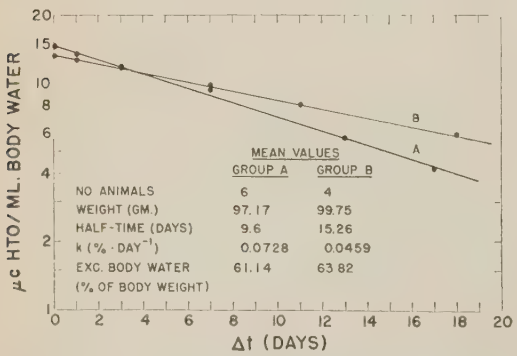

FIG. 1. Change in body water HTO activity in *Dipodomys deserti* as a function of time.

Each 100 g of barley (dry weight) consumed by animals gave rise to 54 g of water *via* biological oxidation of hydrogen(12). The preformed water content of barley available to rats in Group A was approximately 10%. From average food intake for 14 days period, average total daily water intake was 3.9 g/rat. Average daily water intake of animals in Group B (calculated from exchangeable body water and turnover data) was 2.9 g.

Discussion. The value obtained for body water content of *Dipodomys deserti* is in fair agreement with that determined by drying entire animal(2). In a group of 23 animals maintained on dry food only, body water ranged from 62-70% body weight, with a mean of 66% as compared with 62% in our study. This small difference is probably not due to variation in species, since similar values were obtained for *Dipodomys spectabilis* and *Perognathus baileyi* (pocket mouse), which are also members of the family Heteromyidae (2). These investigators found no significant difference between percentage of body water in rats maintained on dry food and on diets of varying water content.

Newly captured animals appear to have a

TABLE II. Individual Values of Parameters of the Water Retention Function.*

Group	Wt (g)	Retention of body water a	k	$T_{½}$ (days)
A	95	14.79	.06376	10.87
	93	14.50	.06637	10.44
	111	13.09	.08183	8.47
	82	17.61	.06746	10.27
	102	14.03	.07719	8.98
	100	14.20	.08002	8.66
Mean	97.17	14.70	.07278	9.62
B	105	12.14	.04536	15.29
	76	17.30	.04485	15.45
	112	11.73	.04816	14.39
	106	12.32	.04508	15.37
Mean	99.75	13.38	.04586	15.13

* $A_t = ae^{-kt}$, where A_t is HTO activity (μc/ml) of body water at any time t; a is HTO activity (μc/ml) of body water at time zero; k is fractional change/day; and t is time in days.

higher percentage of body water than animals in captivity for longer times(2). Captive animals apparently become fatter and less muscular, and amount of body water relative to body weight decreases because fat contains less water than muscle. No statistically significant difference was found between exchangeable body water content of the 2 groups of animals in this investigation, even though determinations were made about 18 and 100 days after their arrival from the desert. The different conditions of relative humidity to which each group was exposed, however, might justifiably preclude any comparison of body water content as a function of time in captivity.

The 10- to 15-day half-time for HTO in the Kangaroo rat is extremely long, as compared with the white rat, which has a half-time of about 4 days. It is interesting also that water retention half-time of the Kangaroo rat (maintained on diet of pearled barley only) is longer than that of the dog, monkey, and man on *ad lib.* water intake. A statistically significant difference ($p = < 0.01$) between mean half-time for each group appears real, in spite of the small numbers of animals. It has been shown by Schmidt-Nielsen *et al.*(3) that there exists a lower limit of relative humidity and temperature (about 10% relative humidity at 75°F) beyond which these animals cannot remain in positive water balance when maintained on a dry diet. The animals in Group B were exposed to very similar conditions, and their increased water retention might represent a response to one or more environmental changes such as light, noise, relative humidity, or time in captivity.

The results of this investigation demonstrate the effectiveness with which the Kangaroo rat conserves water and thereby survives in a xeric environment.

Summary. Mean exchangeable body water content ($\pm \sigma$), as measured by the tritium water dilution method, was 62.37 $\pm$ 2.16% of the body weight for 20 Kangaroo rats (*D. deserti*). When maintained on a diet of pearled barley only, 2 groups of animals showed mean turnover times for exchangeable body water of 13.9 and 22.1 days. This is much longer than that found in the ordinary white rat and in other mammals and presents an adaptation to a xeric environment.

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Preliminary Study of Radioactive Product Obtained from Iodinating Tetracycline. (25709)

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Rall *et al.*(1) and McLeay(2) reported that tetracyclines localize in tumors and bones. This was learned by observing under ultraviolet light the fluorescence in bones and tumors removed from treated animals. The characteristic yellow fluorescence persists in mouse tumor and bone for 10 to 20 days after last injection of one of the tetracyclines. McLeay observed fluorescence in human cancerous tissue removed from patients pretreated with terramycin. While tetracyclines had no effect on growth of tumors, their accumulation in rapidly growing tissues suggested that they might act as carriers for some antitumor diagnostic agent such as radioactive isotope. This paper deals with a product obtained by chemically labeling tetracycline with I^{131}.

Method. Sprague-Dawley rats weighing 150-300 g and spontaneous mammary tumor mice were used. One % sodium iodide solution to block thyroid uptake of detached iodide was given in drinking water. Animals were treated with I^{131} products obtained from reactions designed to iodinate tetracycline. Details of synthesis and characterization of the product will be published later. Animals were injected intraperitoneally once daily for 3 days with 8 mg/kg of product in 4.4% sodium iodide solution. Control animals were injected similarly with equal amount of radioactivity in a mixture of 4.4% sodium iodide, tetracycline and sodium iodide I^{131}. Twenty-four, 48 and 72 hours after last injection the animals were sacrificed, and thyroid, liver, muscle and femur removed. Tumors were also removed from each tumor-bearing mouse. Radioactivity was determined in each tissue by a well-type scintillation counter. Some tissues were biologically assayed(3) for tetracycline-like qualities and fluorescence of bones was studied under ultraviolet lamp.

* Authors are grateful to Dr. Michael Carlozzi of Chas. Pfizer & Co. for generous supplies of tetracycline.

Results. Table I indicates that the radioactive product was taken up preferentially in bones of the animal. Examination under ultraviolet light of femurs split longitudinally indicated that the material was concentrated in the epiphyseal plate area. In rat No. 2 antibiotic activity (0.48 μg/g of tissue) was detected in femur but not in liver or muscle. Radioactivity was associated usually with antibiotic activity, but no antibiotic activity could be detected in bone with specific activity of less than 0.1 cpm/mg of wet tissue. Snell *et al.*(4) reported that the liver accumulates and excretes tetracycline, and it appears next highest in specific activity in our experiments. No antibiotic activity was detected in liver at any time. Muscle had a

TABLE I. Distribution of Radioactivity in Tissues of Young Rats Following Administration of Radioiodinated Tetracycline.

Rat No.	Tissue	Specific activity, cpm/mg wet tissue
1*	Liver	.012
	Femur	.17
	Muscle	.008
2†	Liver	.055
	Femur	.367
	Muscle	.021
3†	Liver	.15
	Femur	.47
	Muscle	.18
4†	Liver	1.9
	Femur	.12
	Muscle	.05
5*	Liver	‡
	Femur	.20
	Muscle	.045
6*	Liver	.009
	Femur	.19
	Muscle	.043
7*	Liver	4.0
	Femur	1.10
	Muscle	.10
Control* (avg of 2 rats)	Liver	.0009
	Femur	.003
	Muscle	.005

* Sacrificed 24 hr after last inj. † Sacrificed 48 hr after last inj. ‡ Not detectable.

TABLE II. Distribution of Radioactivity in Tissues of Mammary Tumor Mice Following Administration of Radioiodinated Tetracycline.

Mouse No.	Tissue	Specific activity, cpm/mg wet tissue	μg of tetracycline/g
1*	Liver	.91	
	Femur	.98	
	Tumor	1.19	
2†	Liver	.052	§
	Femur	.161	8.95
	Tumor	.062	§
3‡	Liver	.05	§
	Femur	.16	5.81
	Tumor	.09	1.4
4*	Liver	.26	
	Femur	.08	
	Tumor	.63	
5*	Liver	.12	
	Femur	.06	
	Tumor	.08	
6*	Liver	.009	
	Femur	.03	
	Tumor	.19	
7* (4 inj.)	Liver	.83	§
	Femur	.39	3.55
	Tumor	.095	§
Control* (avg of 4 mice)	Liver	.012	
	Femur	.020	
	Tumor	.027	

* Sacrificed 24 hr after last inj. † Sacrificed 48 hr after last inj. ‡ Sacrificed 72 hr after last inj. § Not detectable.

low specific activity, which in most instances is of questionable significance.

Table II indicates that mouse tumor contained a higher specific activity than did mouse femur or liver. Muscle from mice showed a low specific activity. Control animals flushed out most of the NaI^{131} within 24 hours.

These data indicate that the radioactive product is a labeled tetracycline or degradation product and that it is inactivated by liver, since considerable radioactivity was measured in liver specimens, but in no case was antibiotic activity demonstrated. In some animals thyroid became radioactive, suggesting that the product was contaminated with NaI^{131}. This was partly alleviated by giving the product in a solution of nonradioactive sodium iodide and giving animals sodium iodide in drinking water thus blocking the thyroid.

These studies indicate a possible new irradiation tool for detection and treatment of tumors. Bone tumors may offer greatest possibilities in utilizing this new tool.

Summary. Attempts to radioactivate tetracycline by iodination reaction have evolved a product which resembles tetracyclines when injected into rats and mice. It is sequestered in general by fast-growing tissues and in particular by tumor, liver and epiphyseal plate region of bone. Liver appears to inactivate and excrete the material.

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Comparison of Effect of Desoxycorticosterone Acetate and Cortisone Acetate on Rat Skeletal Muscle Electrolytes. (25710)

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Recently in a study of differences between hypertensive actions of 2 adrenal steroids, desoxycorticosterone acetate (DCA) and cortisone acetate, the electrolyte composition of eviscerated carcasses of rats injected with one or another of these hormones was determined (1). In animals given the former steroid, while maintained on high sodium intake, an impressive retention of sodium and marked depletion of potassium were noted, findings consistent with previous reports of effects of this compound on skeletal muscle(2,3). In rats injected with cortisone, regardless of sodium content of diet, a severe potassium de-

TABLE I. Allocation of Animals, Experimental Regimen, Duration of Observation, Terminal Blood Pressure Readings, and Body Weights at Sacrifice.

Group	Sodium intake	Adrenals	Steroids, mg/day	Duration exp., days	Terminal No.	Terminal B.P., mm Hg	Terminal Body wt, g
I	High	Excised	DCA 2.5*	21	8	189 ± 10	181 ± 14
II	"	"	Cort. Ac. 2.5†	21	7	195 ± 2	121 ± 12
III	"	"	nil	21	6	132 ± 12	166 ± 15
IV	"	Intact	"	21	6	149 ± 10	159 ± 9
V	Low	Excised	DCA 2.5	14	8	137 ± 6	150 ± 11
VI	"	"	Cort. Ac. 2.5	14	7	195 ± 17	105 ± 5
VII	"	"	nil	4 to 7	7	87‡	104 ± 9
VIII	"	Intact	"	14	6	150 ± 9	150 ± 16

* Desoxycorticosterone acetate kindly supplied by Dr. Kenneth Thompson of Organon, Nutley, N. J.

† Cortisone acetate kindly supplied by Merck & Co.

‡ B.P. obtainable prior to sacrifice in only 1 rat in group. Remaining 6 rats were moribund.

pletion was also observed, though surprisingly not accompanied by any change in sodium, all measurements expressed in terms of fat free dry tissue. However, rats receiving cortisone while on a liberal sodium intake failed to grow, while animals given this steroid while on restricted sodium intake lost as much as a quarter of initial body weight. In contrast, rats receiving DCA gained, as did normal controls, roughly a third as much as their initial weight during same time interval. Inhibiting effect of cortisone on growth was so striking and so different from DCA that it seemed possible that electrolyte disturbances in cortisone groups were a reflection of this growth suppression rather than representing a true shift in electrolyte content of cells. This study therefore focused on analysis of a single tissue from these same animals, skeletal muscle, as opposed to whole carcass. It was hoped that this would permit a more precise comparison between the 2 steroids, though admittedly a more limited one.

Methods. Details of procedures have been described(1). Table I outlines the 8 groups of rats, number in each group, regimens employed, duration of observation, terminal blood pressure recordings and body weights. At sacrifice the right gastrocnemius muscle was removed, animals eviscerated and muscles and carcass saved for analysis as reported previously. The muscles were kept in frozen state until analyzed. Determination of water content was based on weight differences after 48 hours of desiccation in oven at 100-110°F. After desiccation, muscle fat was extracted with ethyl ether in a Soxhlet apparatus. The tissue was then digested with 3 ml of concentrated nitric acid, the digest diluted to 100 ml in volumetric flask containing 1:200 parts of lithium, and sodium and potassium determined by flame photometry.

Results, with standard deviations, are presented in Table II.*

Water content. Muscles from rats injected with cortisone contained significantly less water than muscles from any other group, while those from adrenalectomized rats on sodium restriction (Group VII) contained excess amounts of water.

Amount of fat was so small that comparisons between groups seem of little import. However, muscles of cortisone injected rats on a liberal sodium intake contained the largest amount of fat.

Sodium content. In spite of normal water content, muscles of sodium loaded DCA rats contained more than twice as much sodium as did muscles from other groups. Restriction of sodium intake prevented influx of excess quantities of this cation into muscles of DCA treated rats of Group V. A significant reduction in muscle sodium occurred in sodium depleted adrenalectomized controls. No variance from normal was noted in muscles from either cortisone injected group.

Potassium content. Muscles of sodium loaded DCA rats sustained a highly significant decrease in potassium content to 75% of values obtained in muscles of control ani-

* We are indebted to Dr. Agnes Berger and Miss Helen Perlstein, School of Public Health and Administrative Med., for statistical examination of the data.

TABLE II. Rat Muscle Analysis.

Group	High sodium regimen	Water, %*	Fat, % dry wt	Na^+ meq/100 g FFDT†	K^+ meq/100 g FFDT†
I	DCA + ADRX	74.2 ± .7	4.0 ± 1.0	17.1 ± .9	33.1 ± 1.3
II	Cort. Ac. + ADRX	72.0 ± 1.9	6.7 ± 1.7	8.2 ± .7	42.1 ± 1.9
III	ADRX	74.6 ± 1.6	3.0 ± 1.0	8.3 ± .7	43.8 ± 1.5
IV	Intact controls	74.4 ± .8	1.0 ± .4	8.2 ± .2	44.3 ± .1
	Low sodium regimen				
V	DCA + ADRX	74.8 ± .8		8.4 ± .3	44.8 ± .8
VI	Cort. Ac. + ADRX	72.3 ± 1.1	1.8 ± 1.5	7.6 ± .6	42.8 ± .8
VII	ADRX	76.4 ± .5	1.6 ± 2.0	6.4 ± .5	45.7 ± .9
VIII	Intact controls	74.7 ± .5	3.1 ± 1.4	7.9 ± .5	44.0 ± 1.1

* For each category of measurements, treatment differences were jointly estimated by 95% simultaneous confidence interval following Tukey(6). (This amounts to listing individual treatment differences more strictly than at 5% level.) Values for water, fat, Na^+ and K^+ differing significantly from intact controls on the same regimen are underlined; those of borderline significance are underscored with a broken line.

† FFDT = fat-free dry tissue.

mals. Restriction of sodium intake, as in the DCA injected Group V, prevented development of this abnormality. Muscles from cortisone rats provided with high sodium intake exhibited a lower average potassium concentration of borderline significance. The slight decrease in potassium values in cortisone rats on low sodium did not prove significant on statistical analysis. It seemed possible that these slight changes in potassium were a reflection of dehydration of these muscles; and indeed when potassium was expressed in terms of fat free wet weight rather than dry weight, the content of this cation was normal (112 meq/kg for Group II *vs.* 112 meq/kg for Group IV).

Discussion. That muscles of hypertensive adrenalectomized rats, on regimen of large daily injections of DCA and liberal sodium intake, sustained marked depletion in potassium along with increase in sodium is quite in keeping with similar changes encountered in analyses of eviscerated carcasses of such animals(1), and confirmatory of many previous studies on effect of the steroid on skeletal muscle.

That muscles of hypertensive adrenalectomized rats, on a regimen of large daily injections of cortisone and either low or high sodium intake, contained normal amounts of sodium is in keeping with previously reported analyses of carcasses of such animals. However, values for potassium in muscle, while showing changes in the same direction as in whole carcass, were by no means as impressively reduced. The more strikingly lowered potassium/unit of fat free dry weight in the carcass could be explained if weight losses induced by cortisone involved greater losses from potassium rich tissues such as muscle, than from potassium poor tissues such as skin. This possibility cannot be confirmed by present data, though the observation that normal rats in which intake is restricted to achieve same weight reduction do not show this depletion(4), suggests that the change is not the result of weight loss *per se*. Dehydration of cortisone rats could also contribute to previously noted low carcass potassium. As mentioned above, when muscle data were expressed in terms of fat free wet tissue potassium depletion was no longer evident. When carcass potassium values are similarly expressed in terms of wet weight, depletion of this electrolyte becomes far less impressive, is no longer demonstrable in the group on a restricted sodium intake.

It seems probable that both of above considerations contributed to the low value for potassium obtained in carcasses of cortisone injected rats, neither one necessarily implying reduction in amount of potassium/cell unit. Using the ratio of amount of potassium to nitrogen as a very rough indicator of this last mentioned variable, the figures listed in Table III were computed from previously reported analyses of whole carcass. (In earlier experiments(5), similarly prepared animals given

TABLE III. Carcass Analyses. Additional calculations.

	Sodium meq/kg fat-free wet tissue	Chloride meq/kg fat-free wet tissue	Potassium, meq/g N*
High sodium regimen			
DCA + ADRX	56.8	30.4	1.88 ± .11
Cortisone + ADRX	56.4	36.2	2.13 ± .17
ADRX	46.5	34.0	2.39 ± .17
Intact controls	50.4	33.5	2.29 ± .07
Low sodium regimen			
DCA + ADRX	43.3	31.2	2.41 ± .28
Cortisone + ADRX	46.5	26.5	2.21 ± .31
ADRX	38.0	27.2	2.31 ± .04
Intact controls	38.1	28.0	2.30 ± .20

* See footnote to Table II for methods of statistical analysis.

DCA or cortisone acetate have not shown any alteration in serum urea nitrogen values which might disturb this ratio.) In neither cortisone injected group was the ratio significantly reduced; by comparison the decrease in this value in sodium loaded DCA injected rats of Group I was striking and qualitatively different.

Muscle content of sodium in cortisone treated rats was normal, whether this electrolyte was calculated in terms of dry or wet tissue. However, if figures for sodium in total carcass are expressed in terms of wet weight, the value for this cation now appears definitely elevated (Table III). Since skeletal muscle did not participate in this sodium increase, a possible explanation is that cortisone injected rats lost more weight from those tissues high in potassium than from tissues high in sodium.

It appears that selection of a single tissue, skeletal muscle, to study electrolyte effects of cortisone *vs.* DCA has the advantage of reducing the number of variables which must be taken into account in interpreting the analysis. In this single tissue, cortisone in amounts sufficient to produce severe hypertension in adrenalectomized rats, unlike the steroid DCA, was without effect upon electrolyte composition of muscle when dehydration of tissue was taken into account. These findings provide still further evidence of the different nature of hypertension induced by cortisone from that induced by DCA in the rat.

Summary. 1. Analyses of skeletal muscle from adrenalectomized rats given cortisone acetate in amounts sufficient to induce severe hypertension revealed no impressive alteration in sodium or potassium content from normal, when dehydration of tissue was taken into account. 2. In contrast, skeletal muscle from adrenalectomized rats given comparable amounts of DCA and with comparable degrees of hypertension, showed, as anticipated, marked increase in sodium and reduction in potassium content. 3. In rats injected with cortisone, blood pressure and muscle electrolytes were comparable in groups maintained on a low sodium diet and in groups with liberal sodium intake. In contrast, in rats given DCA, both hypertension and shift in electrolyte content were dependent on high sodium intake; neither was encountered when sodium content of the diet was restricted. 4. Muscles from adrenalectomized rats without steroid subsidy were normal in all respects in groups provided with liberal amounts of sodium but were overhydrated and depleted of sodium in the group sustaining sodium restriction.

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In vitro Synthesis of Progesterone by Swine Corpora Lutea.* (25711)

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Nearly 4 centuries have elapsed since original morphologic descriptions of the corpus luteum were made by sixteenth century anatomists(1). During this interval endocrine relationships have been established between this organ and various aspects of reproduction, including lactation. It is well known that time between periods of estrus in polyestrous animals is dependent upon secretory activity of corpus luteum. However, factors responsible for development, maintenance and regression of the corpus luteum need to be determined because findings of current research argue against the theory that life span of corpora lutea is self-limiting(2). Armstrong and Hansel(3) reported that daily administration of oxytocin to post-puberal non-lactating heifers, during first week of estrous cycle, resulted in marked shortening of the diestrual period. They concluded that observed effects of oxytocin were caused by inhibition of normal corpus luteum function. This report presents a method for *in vitro* evaluation of corpus luteum which may prove useful in elucidation of control of this organ.

Materials and methods. Cross-bred gilts, weighing 225-300 lb, were checked daily with vasectomized boars to determine occurrence of estrus and duration of estrous cycles. Average duration of estrous cycles in gilts is 21 days and pregnancy is 113 days. Animals for pregnancy studies were mated one to 3 times. Ovaries were removed at slaughter, corpora were dissected and placed in chilled containers. Average number of corpora lutea was 14, range 10 to 20/animal. Five hundred to 800 mg of representative tissue slices with maximum thickness of 0.3 mm were transferred to 20 ml beakers containing Krebs Ringer bicarbonate buffer medium (pH 7.4) with 200 mg % glucose at 1 ml/100 mg tissue. Slices were incubated 2 hours in Dubnoff Metabolic Shaking Incubator at 37.5°C under atmosphere of 95% O_2 and 5% CO_2. Gas flow was 4 c.f.h. Preliminary work indicated that progesterone was synthesized by luteal tissue throughout incubation of 6 hrs. Control samples for determination of initial endogenous progesterone concentration were prepared at the same time as those to be incubated but were extracted immediately with 95% ethanol. Following incubation, tissue was homogenized in medium and extracted 3 one hour periods with 75 ml of 95% ethanol at 75°C. Pooled ethanol extracts were evaporated under reduced pressure at 60°C for column adsorption chromatography. A column was prepared by pouring a suspension of 4.5 g of aluminum oxide (Merck 71707) in n-hexane,† into chromatographic tube 10 mm diameter and 300 mm long. The residue was dissolved in 20 ml of n-hexane and applied to the column, followed by two 15 ml rinses. Column development and subsequent countercurrent distribution of eluate fraction containing α, β unsaturated steroids followed procedure of Loy *et al.*(4). Solutions contained in center 5 funnels, theoretically containing 93% of progesterone introduced in first funnel, were combined and subjected to paper partition chromatography to preclude possibility of contaminating substances. The solvent systems were those of Bush(5) and Savard(6). Area of chromatogram containing progesterone, as identified by ultraviolet scanning and a reference chromatogram, was eluted and concentration calculated by equation of Allen(7) from observed absorbancies at 230, 240 and 250 mμ. A standard curve was prepared from absorbancy readings of progesterone in 95% ethanol ranging from 1 to 30 μg/ml. Molar absorbancy index (ϵ) was 16,700 at 240 mμ. Average recovery value of 84.7% was obtained by adding 25 to

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† Technical grade, Phillips Petroleum Co., Bartlesville, Okla.

TABLE I. *In Vitro* Synthesis of Progesterone by Swine Corpora Lutea.

Day	No. of animals	Avg wt total luteal tissue per animal, g	Progesterone concentration, μg/g			Avg luteal progesterone per animal, μg
			Initial	After 2 hr incubation	Increase	
Of cycle						
4	3	1.11	21 ± 5.4*	158 ± 17.4*	137	23
8	3	5.32	40 ± 4.3	113 ± 17.7	73	213
12	4	5.49	61 ± 4.3	135 ± 13.3	74	335
16	3	4.20	74 ± 3.1	146 ± 22.9	72	311
18	3	1.40	0	0	0	0
Of gestation						
16	3	5.82	82 ± 3.0	114 ± 12.1	32	477
24	4	5.18	84 ± 4.7	145 ± 24.1	61	435
48	4	5.50	105 ± 7.6	141 ± 6.4	36	578
72	4	5.30	68 ± 4.3	106 ± 14.5	38	360
96	3	5.61	52 ± 3.1	98 ± 8.6	46	292
102	3	5.71	21 ± 9.2	68 ± 13.9	47	120

* Mean ± S.E.

100 μg of progesterone to 8 different tissue samples ranging 400 to 600 mg. Total hormone content of these samples ranged from 55 to 206 μg.

Results. Data in Table I show average weights of luteal tissue/animal and progesterone concentrations, prior to and following 2 hour incubation period. Slices of swine corpora lutea were capable of synthesizing progesterone *in vitro* whereas homogenates of the organ did not produce the hormone under similar conditions. Furthermore, little if any, progesterone diffused into incubation medium from the tissue. Enzymic nature of the reaction is shown by complete inhibition of synthesis when luteal tissue was heated at 95°C for 30 minutes. Intermediate rates of biosynthesis were observed at room temperature (28°C). For example, tissue which synthesized 44 μg/g during 2 hr incubation synthesized 25 μg/g during 6 hr at room temperature. Similarly, tissue synthesizing 70 μg/g during incubation synthesized 12 μg/g during 2 hr at room temperature.

Pregnenolone, a probable precursor in biosynthesis of progesterone, was added to medium in concentrations ranging 117 to 794 μg/g of luteal tissue. Progesterone synthesis increased to 208% above incubated controls in samples containing pregnenolone. Maximum conversion of pregnenolone to progesterone during 2 hr incubation was 74%. DPN also stimulated progesterone synthesis. Concentration of progesterone was increased by 89 μg/g when 6 μM of DPN was added. Addition of 500 μg of pregnenolone to another aliquot of tissue increased concentration 95 μg/g. Addition of both 500 μg of the precursor and 6 μM of DPN to another tissue aliquot increased progesterone concentration 235 μg/g.

Discussion. In evaluating functional aspects of the corpus luteum it is desirable to consider briefly morphologic development and retrogression of the organ(8). During week following ovulation, corpora lutea attain a diameter of 8-10 mm. If the animal is pregnant there is further growth until an average diameter of 10-11 mm is reached. Histologically it has not been possible to distinguish between corpora of the active luteal phase of non-fertile cycle and those of early pregnancy. At approximately day 16 of cycle, a sudden change occurs in appearance of corpora lutea in non-pregnant animals. By day 18 the diameter decreases to 6 mm and color changes from pink of active capillary circulation to whitish of scar tissue indicating retrogressive changes in the nature of fibrous involution. Eventually all that remains of the site of an ovarian follicle, and subsequently a corpus luteum (of either a nonfertile cycle or of pregnancy), is a small mass of scar tissue, a corpus albicans.

Concentration of progesterone in luteal tissue increased from 21 μg/g at day 4 to 74 by day 16 of the cycle. However, at day 18 (late diestrum) no detectable progesterone was present. Increase in hormone concentration was found after 2 hr incubation with the ex-

ception of tissue from day 18. The greatest increase, 137 μg/g, was observed in luteal tissue from animals at day 4 of cycle. This increase was nearly twice that found in tissue from animals at days 8, 12, and 16. Initial progesterone concentrations in corpora lutea from animals at days 16, 24 and 48 of gestation were higher than those observed in tissue from gilts during the cycle. However increase in progesterone concentration following incubation was greater in corpora lutea from non-pregnant than from pregnant animals. A difference of 8 μg/g between mean initial progesterone concentration at day 16 of cycle and day 16 of pregnancy, is not statistically significant at 5%. However, increases in concentration following incubation within each of these 2 stages are statistically significant at 5%. Data summarized in Table I indicate a decrease in hormone concentration during latter part of pregnancy. The average quantity of luteal progesterone/animal was 23 μg at day 4 of cycle, 335 μg at day 12 and 0 by day 18. The greatest amount of luteal progesterone/animal during pregnancy was 578 μg at day 48. This quantity decreased to 120 μg by day 102. Morphologic development and regression of the corpus luteum parallel capacity of luteal tissue to synthesize progesterone as indicated by results of this study.

Kimura and Cornwell(9), using ovariectomized rabbit assay of Allen(10), reported a similar trend for progestin concentration in corpora lutea of pregnant sows during last stages of gestation. They reported a concentration of 50 μg/g in sows pregnant 40 to 75 days. Loy *et al.*,(11) investigated progesterone concentration in corpora lutea from gilts slaughtered at 70th day of gestation. Hormone concentration ranged from 20 to 91 μg/g. Their data are in agreement with the range of 36 to 76 μg/g reported by Allen(10). Elden(12) using the Allen assay found a range from 41 μg/g during early pregnancy to 16 μg/g in late gestation in the sow. Gawienowski(13) used a chemical procedure and reported progesterone range of 5.9 to 37.9 μg/g of corpora lutea from sows 55 days pregnant. Short(14) reported progesterone concentration in plasma of sows during luteal stage of cycle and at day 90 of pregnancy as 0.83 and 0.34 μg/100 ml respectively. No progesterone was detected 4 days postpartum.

Summary. A method is described for investigation of *in vitro* synthesis of progesterone by swine luteal tissue Heating tissue for one half hour at 95°C inhibited *in vitro* production of hormone. Addition of pregnenolone and DPN to tissue medium increased progesterone synthesis. Average weight of luteal tissue/animal was 1.11 g at day 4 of estrous cycle, 5.49 at day 12 and 1.40 at day 18. Average weight of corpora lutea during gestation was relatively constant. During estrous cycle, concentration of progesterone in luteal tissue increased from 21 μg/g at day 4 to 74 μg/g by day 16. At day 18 no detectable hormone was present. An increase in hormone concentration was found after 2 hr incubation in Krebs Ringer bicarbonate buffer medium except with tissue from day 18; greatest increase was observed in tissue from day 4 of cycle. During gestation, initial progesterone concentrations in luteal tissue from animals at days 16, 24 and 48 were higher than those in non-pregnant animals. However, increase in progesterone concentration following incubation was less in corpora lutea from pregnant than from non-pregnant animals.

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Relationship of Hepatic Uptake of Free Fatty Acids to Plasma Concentration.* (25712)

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The metabolism of free fatty acids (FFA) presents an intricate picture to investigator who seeks to relate plasma FFA concentration to cellular handling of this material. Net FFA plasma concentration at any instant must equal the algebraic sum of production, destruction, storage, or conversion. These parameters in turn may be regulated by local mechanisms at cellular level, or, vary each in a particular direction under the influence of endocrine or nervous control. Certain tissues release into, while others remove FFA from circulation. Gordon(1) found both myocardial and visceral extraction of FFA in unanesthetized patients. Liver and heart of dogs remove FFA(2,3,4,5,6) while adipose tissue has been described in dogs and humans(1,2,3,4) as supplying FFA to plasma. The action of various normally secreted hormones, as well as the autonomic nervous system on FFA plasma levels has been widely investigated (7,8,9,10,11). The relation of cellular output to cellular utilization is of prime importance in depicting the integration of forces acting to set FFA plasma concentration at a particular level. Cellular uptake of FFA might be considered more tangibly if one studies the reaction of such cells to *in vivo* changes in plasma FFA concentration, this alteration brought about without stimulating the output mechanism in adipose tissue. The present investigation reports canine hepatic reactions to changes in portal vein FFA content induced by infusion of FFA-loaded serum into portal tributary.

Methods. Adult mongrel dogs were anesthetized with intravenously administered pentobarbital sodium (.03 g/kg). Hepatic vein samples were obtained from polyethylene cannulae placed in these vessels through jugular vein. The position of each cannula was checked before experiment was begun, by manual recognition through midline abdominal incision. A smaller catheter was then passed through mesenteric vein until its tip closely approximated junction of portal vein with liver. From this cannula, samples representing mixed portal blood were obtained. For sampling A-V differences in leg tissue cannulae were placed in femoral vein and artery through small side branches. A large bore cannula for rapid infusion of serum into portal vein was then inserted a short distance into a more caudal mesenteric vein, but not allowed to enter the portal vessel itself. All cannulae were maintained patent by a slow infusion of physiological saline. To obtain quantitative data regarding liver behavior, estimated hepatic plasma flow (EHPF) was determined by BSP dilution method of Bradley *et al.*(12). A priming dose of 50 mg of dye was administered followed by constant infusion rate of .060-.070 mg/kg/min into a vein of upper extremity. After arterial BSP concentrations had stabilized (usually about one hour) samples were drawn into chilled syringes and prepared for determination of FFA and glucose concentrations and EHPF. Free fatty acids were determined in duplicate analyses by Dole's method(7); Nelson's colorimetric adaptation of Somogyi's glucose determination was carried out in quadruplicate determinations(13). Serum was prepared by obtaining serum from donor dog the previous day, and adding to a particular volume enough sodium oleate so that the concentration added would be at least 2 mg/ml serum. After loading with oleate, the serum stood overnight in the cold. Just before infusion, serum was filtered for removal of particulate matter. A volume of serum from original pool was treated in an identical fashion except that sodium oleate was not added. This serum served as "control serum" infused prior to infusion of loaded serum. To both sera was added an amount of BSP which gave a concentration equal to portal BSP level at time

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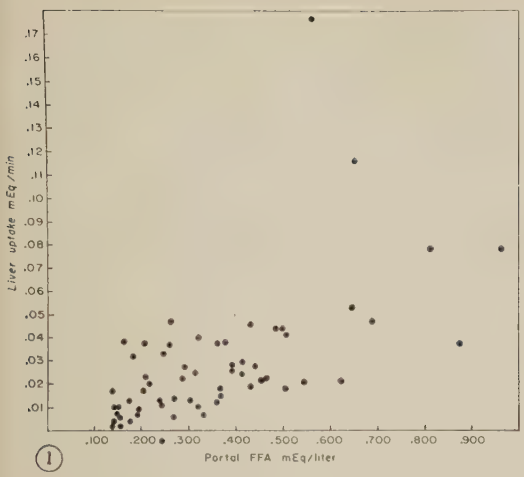

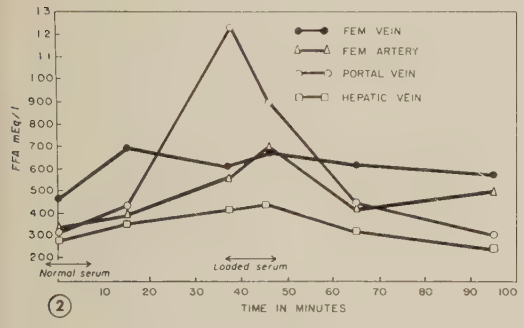

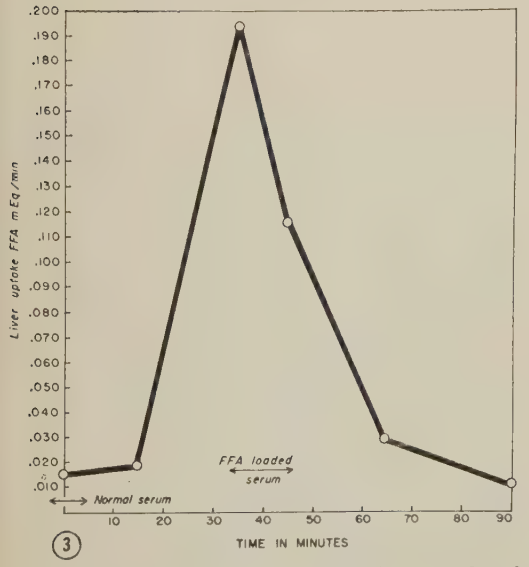

FIG. 1. Scattergram of portal FFA *vs* uptake of FFA by the liver.

FIG. 2. Effect of increased portal FFA concentration on plasma FFA levels.

FIG. 3. Hepatic FFA uptake as a function of portal concentration.

of infusion, as determined just before infusion was started.

Results. Analysis of many studies on liver uptake of FFA in dogs has given us the impression that this organ has a tendency to remove a quantity of FFA in proportion to portal concentration. A scattergram of portal FFA levels *versus* liver uptakes is presented in Fig. 1. Each point was obtained from a different animal in anesthetized but otherwise untreated state. No such suggestion of proportionality can be obtained if one plots EHPF *versus* liver uptakes of FFA in the same animals. In another group of dogs, portal FFA concentration was increased in each animal by rapid infusion of serum rich in sodium oleate. Fig. 2 shows FFA concentrations existing in hepatic, portal and femoral veins as well as femoral artery during sampling periods in 1 experiment. Fig. 3 relates liver uptake of FFA as a function of portal FFA concentration in the same animal. Liver uptake values were obtained by multiplying FFA differences between portal and hepatic vessels by estimated hepatic plasma flow. Data on both FFA, glucose and EHPF of all experiments are presented in Table I.

Results show that intraportal infusion of normal serum at rate employed for experimental serum (8-14 ml/min) had little effect on EHPF or, portal hepatic FFA differences, and values for 2 control determinations (first 2 samples of each experiment) are almost identical for absolute FFA hepatic removal. Rapid infusion of serum rich in FFA (Na oleate) however, produced expected increases in portal FFA presentation to the liver; at the same time, hepatic vein concentration of this substance increased very little, thus creating vastly elevated portal-hepatic differences.

Discussion. In discussing sequence of events occurring in humans given glucose and insulin, Gordon(1) hypothesized that transport of fat is controlled primarily by adipose tissue, and that glucose injection causes first a decrease in FFA liberation from depots; this in turn lowers arterial levels, and lastly there is cessation of extraction by other tissues. This implies, as suggested by Dole(14,15) that cells will remove FFA in a passive man-

TABLE I. Results of Portal Infusion of FFA-Loaded Serum on FFA Uptake and Glucose Output by the Liver.

Sample	1	2	3	4	5	6	7
Min. from infusion		+15				+5	+20	+40
Dog 319 (20.8 kg)	Control serum		—8 cc/min.—		14 cc/min.		
△ PH FFA*	.110	.099	.470	.349	.910	.124	
" glucose†	33	—	21	30	30	50	
EHPF‡	410	400	280	310	374	250	
Hepatic glucose output§	135	—	59	100	111	125	
" FFA uptake‖	.045	.040	.135	.108	.338	.031	
Dog 320 (15.5 kg)	Control serum		14 cc/min.	10 cc/min.			
△ PH FFA	.049	.067	.819	.462		.113	.061
" glucose	7	22	36	42		44	13
EHPF	310	293	236	252		262	175
Hepatic glucose output	22	64	82	105		114	23
" FFA uptake	.015	.019	.194	.116		.029	.011
Dog 322 (19.8 kg)	Control serum		8 cc/min.	10 cc/min.			
Arterial FFA	.302	.318	.406	.592	.410	.316	
Venous "	.403	.427	.597	.630	.501	.705	
Hepatic "	.334	.290	.395	.600	.245	.290	
Portal "	.278	.308	.524	.809	.336	.333	
Arterial glucose	79	87	89	88	112	99	
Venous "	84	86	88	92	104	100	
Hepatic "	127	120	158	153	116	131	
Portal "	85	82	104	109	101	98	
△ PH FFA	–.056	.018	.129	.209	.091	.043	
" glucose	42	38	54	44	15	33	
EHPF	440	470	425	495	550	350	
Hepatic glucose output	184	178	232	220	82	113	
" FFA uptake	–.024	.009	.055	.105	.050	.043	

Serum infusions (for loaded and control serum) are shown above the sample columns in each experiment.

* meq/l. † mg %. ‡ ml/min. § mg/min. ‖ meq/min.

ner, depending on plasma concentration and that FFA regulatory mechanisms act on cells which produce FFA. Actually, our work is, in a fashion, a test of this hypothesis, but the reverse condition (increasing FFA liberation as simulated by injection) is used as the first change. It would, if sequence is correct, follow that (1) increased FFA liberation leads to (2) increased plasma levels and as a result (3) increased removal by the liver (and perhaps other organs) takes place. Indeed, an increased removal was demonstrated.

In Dogs 319 and 322, the third samples were obtained while infusion rate was about 8 ml/min of loaded serum; during sample 5 the rates increased to 14 and 10 ml/min respectively. Sample 3 from Dog 320 (portal FFA 1.23 meq/l) represented a portal infusion rate of 14 ml/min, and sample 4 an infusion rate of 10 ml/min (portal FFA .900 meq/l). Thus, different portal concentrations were achieved by merely adjusting rate of portal infusion. Within 20 minutes after infusions were stopped, both venous and arterial levels had fallen to control levels, or, slightly below. Femoral V-A differences were decreased during infusion of loaded serum.

It appears that amount of FFA removed by liver is a function of concentration presented, implying that a local mechanism may exist in hepatic cells which can vary the amount of FFA removed from portal plasma.

Sudden elevation of FFA concentration appeared to have little effect on glucose output by liver, as compared to injection of control serum. The slight rise in glucose levels may have been the result of action on liver of amines introduced with injected sera, since preparation of serum might release serotonin from platelets, or, adrenalin may be released

during exsanguination of the donor animal.

Summary. Behavior of liver to changes in portal concentration of FFA was studied. Rapid infusion of serum containing large amounts of FFA into portal veins of dogs caused increased hepatic uptakes of this metabolite. Uptake of FFA was a function of portal concentration. Liver cells behave in a passive manner to fluctuations in serum FFA concentration, and local hepatic mechanisms are responsible for changing degree of FFA uptake.

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Growth of SE Polyoma Virus in Primary and Established Cell Cultures.* (25713)

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The SE polyoma virus multiplies *in vitro* with cytopathic effect (CPE) in primary tissue cultures from mouse embryo(1) and monkey kidney(2) and in an established cell line (P388 D_1) derived from a murine lymphoma(3), but fails to grow in rat embryo tissue cultures(4). This study reports results of experiments to determine if polyoma virus multiplies in other primary and established cell cultures to determine further the host range spectrum of this virus *in vitro*.

Materials and methods. The SE polyoma virus (strain 210-22) was obtained from Dr. Bernice Eddy and propagated in mouse embryo tissue cultures. Presence of virus was determined by infectivity titrations as described previously(1). Cultures were observed 3 weeks for CPE and fed at weekly intervals. $TCID_{50}$ end points were calculated according to method of Reed and Muench (5). Four different tissue culture systems were used: mouse embryo cells, L cells, rabbit embryo cells and HeLa cells. Tissue culture tubes of mouse embryo cells were prepared with trypsin-dispersed cells from primary cultures of 14- to 18-day-old mouse embryos by modification of trypsin digest method of Youngner(6). Eagle's medium(7) modified by using Hanks(8) BSS and 10% bovine fetal serum was used as growth medium. Medium consisting of 8 parts Eagle's medium with double concentration of amino acids and vitamins, one part tryptose phosphate broth and 2% horse serum was used as maintenance medium. L cells were maintained in Hanks BSS with lactalbumin hydrolysate 0.5%, yeast extract 0.01% (HLYE) and 2% horse serum. In one experiment, an additional set of cultures of L cells was maintained in syn-

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TABLE I. Growth of SE Polyoma Virus.

Tissue	Inoculum	Virus content 18 hr Fluids	18 hr 3rd washing	1 wk Fluids	1 wk 3rd washing	2 wk Fluids	2 wk 3rd washing	3 wk Fluids
Mouse embryo	$10^{4.5}$*	—	—	$10^{7.0}$	$10^{5.1}$	$10^{7.3}$		
L cells HLYE†	"	—	—	$10^{5.2}$	$10^{3.1}$	$10^{5.6}$	$10^{2.6}$	$10^{5.8}$
" " C‡	"	—	—	$10^{4.8}$	$10^{3.3}$	$10^{4.6}$	"	$10^{4.8}$
HeLa	"	—	—	$10^{3.8}$	$10^{2.1}$	$10^{3.4}$	$10^{1.6}$	$10^{2.8}$
Mouse embryo	$10^{5.3}$	$10^{4.5}$	$10^{4.5}$	$10^{7.5}$	$10^{6.0}$	$10^{8.0}$	$10^{6.5}$	$10^{7.0}$
L cells HLYE†	"	"	$10^{2.3}$	$10^{4.2}$	$10^{4.6}$	$10^{3.5}$	$<10^{1}$	$10^{3.7}$
Rabbit embryo	"	$10^{5.3}$	$10^{2.2}$	$10^{2.8}$	$<10^{1}$	$10^{1.2}$	"	$<10^{1}$
HeLa	"	$10^{4.2}$	$10^{2.3}$	$10^{2.5}$	10^{1}	"	"	"

* $TCID_{50}$. † Hanks' BSS lactalbumin yeast extract. ‡ Synthetic medium.
—, not done.

thetic medium(9) containing only amino acids and water-soluble vitamins. HeLa cells were maintained in HLYE with 2% horse serum. Rabbit embryo cells derived from primary culture of trypsinized whole rabbit embryo were maintained in the same media as mouse embryo cells. To determine capacity of different cells to support propagation of polyoma virus, 6 culture tubes of each cell system were inoculated with approximately 10^5 $TCID_{50}$ of the virus. Four tubes for each cell system served as controls. At daily intervals tissue cultures were observed microscopically for CPE. At weekly intervals fluids were removed, pooled and stored at –70°C for titration; each culture was washed 3 times with 2 ml of Hanks BSS and pooled fluids from third washing were taken for titration. In a second experiment, procedure was modified as follows. After infection of cultures, an 18-hour period of incubation was allowed for adsorption. Fluids were removed, pooled and stored for titration. Each tube was washed 3 times with 2 ml of BSS, and the fluid of third washing was pooled and stored for titration. Tubes were refed with proper medium and incubated at 37°C as before.

Results of experiments to determine ability of different cell systems to support virus growth are summarized in Table I.

In Exp. 2 it was evident that during the 18-hour period allowed for attachment, about 90% of the virus was adsorbed in all cell systems except rabbit embryo tissue, in which no decrease in virus titer of fluid was detectable. Active multiplication of the virus was evident in both experiments when mouse embryo tissue was used, the virus reaching titers as high as 10^8 $TCID_{50}$/ml. Multiplication of virus did not take place in HeLa and rabbit embryo cells. The moderate rise of virus in fluids bathing HeLa cells after washing with BSS in the first experiment appeared due probably to slow release into supernatant fluids of virus associated with the cells. In the first experiment with L cells, active multiplication of virus took place, since amount of virus in samples taken at any week was higher than that of inoculum. In the second experiment such a clear-cut rise of virus titer did not occur. However, the virus released into fluids at the end of each week was much greater than that seen with HeLa and rabbit cells.

Microscopic observation of both control and infected tubes of the cell systems revealed typical CPE(1) only in mouse embryo cell cultures. Rabbit embryo, HeLa and L cells showed no differences between infected and control cultures.

In the first experiment, CPE in mouse embryo cultures was complete at end of second week and the tubes were discarded. In the second experiment, cultures could be followed for the 3-week period.

Discussion. Results of experiments in which different cell systems were inoculated with polyoma virus illustrate a clear difference in their ability to support virus growth. Mouse embryo cells, as shown by Eddy *et al.* (1), are suitable for propagation of the agent. Rabbit embryo and HeLa cells do not support multiplication of the virus. L cells seem to support growth of polyoma virus but at a

slower rate than mouse embryo cells. Failure to reach a comparable rate of multiplication may be a characteristic of this cell line or could depend on nutritional conditions in our experiments, since fluids were changed only weekly and a low concentration of horse serum was used. Furthermore, media used for L cells contained less bicarbonate than that employed with mouse embryo cells, and it was recently reported(10) that virus yield is dependent on bicarbonate concentration of the medium.

Through analysis of host-virus relationships with polyoma virus so far studied, it appears that capability to induce tumors in animals is not necessarily correlated with ability to multiply *in vitro* in cells from the same animals nor with ability to produce CPE in the same cells. Moreover, a correlation does not exist between ability to multiply and CPE. For instance, polyoma virus induces tumors in mice(11) and multiplies in mouse cells *in vitro* with CPE(1). On the other hand, in rats(12) and rabbits(13) tumors are produced, but neither virus growth nor CPE takes place *in vitro*. In hamsters, tumors are induced(14), multiplication of virus takes place *in vitro*, but no CPE is detectable(15). Then, when multiplication *in vitro* occurs, it may result in killing of infected cells (CPE) or in production of virus without cell degeneration, as shown in our experiments with L cells and by Vogt and Dulbecco in hamster tissue cultures(15). Therefore, 3 different kinds of relationships between polyoma virus and cells exist: malignant transformation, multiplication with CPE, and virus production with survival of infected cells. Whether the type of response depends on predetermined and genetic characteristics of cells or on modifiable conditions is now under investigation.

Summary. Propagation of SE polyoma virus in 4 different cell systems *in vitro* has been studied. Mouse embryo cells support multiplication of this virus with CPE. Growth occurs to a lesser extent in L cells without CPE, while it does not take place in HeLa and rabbit cells.

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Biochemical Polymorphisms in Animals: Haptoglobins and Transferrins. (25714)

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Ford(1) has defined polymorphism as "the occurrence together in the same habitat of 2 or more discontinuous forms of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutation." Biochemical polymorphic traits of men

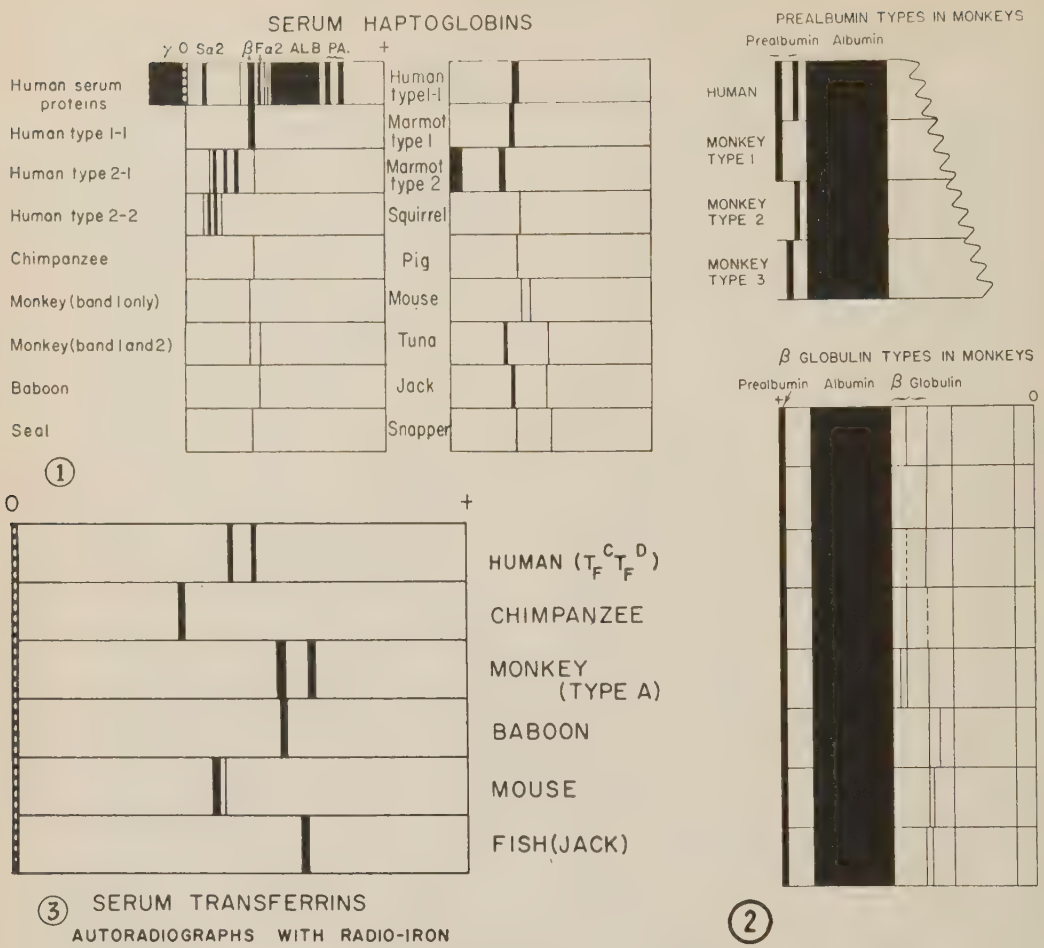

FIG. 1. Diagrams of positions of haptoglobin bands in several animals (see Table I for numbers studied). The excess hemoglobin band is not shown. Position of fast-moving bands in 3 fish is not consistent. Relative positions of human serum proteins shown in the first panel. γ, gamma globulin; O, origin; Sa2, slow alpha-2-globulin; ALB, albumin; PA., pre-albumin; +, positive pole. Nomenclature is that of Smithies(3).

FIG. 2. Diagram of monkey serum protein pattern after starch gel electrophoresis in borate buffer and amido black staining (see text). Origin is on right and positive pole to left. Gamma globulins are not shown. The 8 different patterns of beta globulins are indicated. Autoradiographs made with serum containing radioiron to demonstrate position of transferrin bands were made, but are not shown in order to conserve space. Radioiron binds to each of the β-globulin bands, including the slow one in type G, but not to the slow moving band in type E. The polymorphism in the position of the fast-moving prealbumin is also shown diagrammatically. Iron does not bind to the prealbumin(7,12).

FIG. 3. Starch gel electrophoresis (tris buffer) of animal and human serum which has been equilibrated with radioiron (see text). Diagram of contact print of autoradiograph showing relative position of transferrin bands.

and animals are being discovered with increasing frequency. In the present paper preliminary data on 2 such systems are presented. Haptoglobins are a family of serum proteins which bind hemoglobin(2). Smithies(3) has shown by means of starch gel electrophoresis that there are 3 main haptoglobin types determined by 2 autosomal allelic genes Hp^1 and Hp^2 (Fig. 1). The presence of polymorphism for beta-globulins has been shown in some human populations(4), cows(5), sheep(6), and other animals(7) by means of starch gel electrophoresis. Beta-globulins appear equivalent to "transferrin," the serum protein which binds iron. In humans, transferrins are genetically controlled at a single locus, Tf, by at

least 3 alleles Tf^B, Tf^C and Tf^D(4).

Methods. Starch gel electrophoresis was by the method of Smithies(3). All studies were done using both conditions for serum proteins (borate buffer pH 8.6-8.9) and discontinuous (tris) buffer system of Poulik(8). Haptoglobins were determined by adding approximately 120 mg/100 ml of human hemoglobin solution to serum and staining for hemoglobin bound to the haptoglobins using benzidine and hydrogen peroxide after completion of electrophoresis. Fe^{59} (as $FeCl_3$) was obtained from Abbott Labs (Oak Ridge, Tenn.). Aliquots of aqueous solution were added to serum and equilibrated at 4°C for 24 hours. Final concentration of Fe^{59} in serum was approximately 3×10^{-5} molar. For radio-iron studies, the gel was split in half, one half used for autoradiography, and the other stained for protein with amido black.

Results. Haptoglobin patterns of 208 lower primates have been studied. These include 170 monkeys (*Macaca mulatta*), 4 chimpanzees (*Pan toglodytes*) and 34 baboons (*Papio*). In none of these was a pattern resembling a human type 2-2 or 2-1 seen. All of them had a band in approximate position of type 1-1 haptoglobin (Fig. 1). In approximately ⅓ of the monkeys, there was a second band (band 2) traveling faster than the common one. After addition of excess of hemoglobin, migration of band 2 became slower and merged with the slower moving band. A similar phenomenon is seen in serum of seals(7). Samples of sera, drawn at one month intervals, were available from 10 monkeys. Position of haptoglobin band 1 was identical in both specimens; the fast-moving band (band 2) was, however, absent or considerably decreased in some animals in the second specimen. This suggests that this band is not persistent during life. Fast-moving bands, similar to monkey band 2, were seen in chimpanzee, baboon, and some other animals. None of the other animals (Table I) tested had a pattern similar to human 2-2 or 2-1, but many had a haptoglobin band with approximately the same migration as human type 1-1. Several unusual varieties were seen (*i.e.*, marmot type 2) and some animals had bands migrating faster than the haptoglobin type 1-1 band. This was in nearly all cases excess hemoglobin, but fast-moving haptoglobin bands could not be ruled out in every case, since some sera used were hemolyzed. Mice had a band in the position of human 1-1 band as well as a fast-moving band. There was some variation from strain to strain in position of fast-moving bands and in amount of hemoglobin bound.

TABLE I. Animals Studied. Numbers examined for haptoglobins are also given. For further information on animals 4, 5, and 6, see reference(12).

Species	No. studied
1. Chimpanzee *(Pan toglodytes)*	4
2. Monkey *(Macaca mulatta)*	170
3. Baboon *(Papio)*	34
4. Seal *(Callorhinus ursinus)*	30
5. Arctic marmot *(Marmota caligata broweri)*	4
6. Ground squirrel *(Citellus parryii barrowensis)*	8
7. Domestic pig *(Sus scrofa)*	5
8. Mouse *(Mus musculus)*	10
9. Tuna (fish) *(Cymnosarda nuda)*	1
10. Jack (fish) *(Caranx sexfasciatus)*	3
11. Snapper (fish) *(Lutjanus vaigiensis)*	1

Beta-globulins. In a study of sera of 143 monkeys, several distinct beta-globulin patterns have been found (Fig. 2). The difference between type B and type C seems a quantitative one, and these 2 types have been grouped together. Distribution of types was as follows: type A, 51; type B and C, 67; type D, 8; type G, 5; type H, 7; type J, 4. The distinction between types J and H was sometimes difficult. In addition, there was one sera of type E. A diagram of autoradiograph of monkey type A, compared to human transferrin type CD (genotype Tf^C Tf^D) is shown in Fig. 3. A description of monkey autoradiographs is given in caption of Fig. 2. The same patterns were seen using borate or tris buffer, but better resolution was obtained with the latter, particularly if the gel buffer was diluted to half strength.

The radioiron binding of 4 chimpanzees, 7 baboons, 6 strains of mice, and a fish (snapper) were also studied (Fig. 3). No intraspecies variations were seen. There is a single band in chimpanzees, baboons, and fish which, in chimpanzees and mice, migrated slower, and in monkeys, baboons, and fish, migrated faster than human transferrins. These studies do not rule out a polymorphism in these species, and Dr. J. Buettner-Jansuch

has kindly informed us that he observed variation in baboon beta-globulin patterns.

There are 3 positions of pre-albumin band of monkeys (Fig. 2), and this may also constitute a polymorphism(7). This protein binds radiothyroxine(12).

Discussion. In comparing inherited proteins in different species, it cannot be assumed that proteins that have the same mobility on starch gel electrophoresis, or that bind the same material (*i.e.,* hemoglobin and iron) are the same substances. Although such proteins may be very similar, it is necessary to compare them more explicitly by physical and chemical means before their equivalence can be assumed. Such studies are in progress.

Haptoglobin polymorphism in humans does not occur in monkeys studied, all those tested having a protein with migratory properties similar to that of human type 1-1 haptoglobin. This implies that the Hp^2 gene is rare in monkeys and probably also in other animals tested. In terms of some of the concepts on development of polymorphisms(9), this may indicate that if a mutation to the Hp^2 gene occurred in monkeys, it offered no advantage in internal and external environment then obtaining, and it did not increase in the population in subsequent generations. On the other hand, when the human gene pool experienced this mutation, conditions favored its spread in the population, and it increased in frequency until balanced by other forces.

Examples analogous to this have been seen in other polymorphic systems, such as the trait associated with urinary excretion of beta aminoisobutyric acid (BAIB)(10). On the other hand, polymorphisms present in humans have been found to exist in lower primates. Fisher, Ford, and Huxley have shown that dimorphism for taste of phenylthiocarbimide is present in chimpanzees and probably other primates(11), and it appears to be present in monkeys (*Macaca mulatta*) as well (Blumberg, unpublished observation).

In transferrins, polymorphism exists in primates but appears to involve proteins with different physical properties than human transferrins. It will be of interest to compare these proteins chemically and physically and to determine the evolutionary significance of their differences.

Summary. Two genetic polymorphic systems, haptoglobins and transferrins, have been studied in various animal species. Haptoglobin polymorphism, present in human populations, has not been found in other primates so far tested. A polymorphism for transferrins exists in monkeys, but it includes proteins different from those found in human polymorphism.

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Toxicity Studies on Dibutyl- and Diamyl-Hydroquinone. (25715)

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Carlson and Brewer(1) reported on chronic toxicity of the antioxidant, hydroquinone. The derivatives of HQ, reported here, are even less toxic than the parent compound. High, Woods, and Wilson(2) gave mono-*tert*-butylhydroquinone (10 mg/day) to rats by mouth for 20 days to study effect on utilization of carotene. They reported no significant effect on growth and mentioned no adverse symptoms. Fassett and Roudabush(3) injected mono- and 2,5-di-*tert*-butylhydroquinone (DBH) intraperitoneally into growing rats 5 times weekly for 4 weeks, the highest daily dosage being 200 mg/kg or about half the LD_{50}. The highest level of mono compound interfered with growth, while there was no such response to 2,5-derivative. Oral administration of DBH in comparable dosage to dogs for 2 months, and to rats for an undisclosed time, produced no evidence of cumulative action. Effects of DBH and 2,5-di-*tert*-amylhydroquinone (DAH) to chickens has been reported(4). Acute oral administration of DBH produced toxic symptoms at levels of 2 g or more/kg, and 50% mortality when 10 g/kg was given. Continued administration of 0.25% in feed led to nervous symptoms, increased mortality, decreased growth, and reduced egg production and hatchability. On the other hand continued feeding of DAH produced no more than a slight impairment of growth. The present study deals with oral toxicities of DBH and DAH to rats, with skin toxicity and sensitization, and, briefly, with effects on hair pigmentation.

Methods. Chronic toxicity tests were made with weanling albino rats from our colony, incorporating test substances into diet and feeding *ad lib.* for considerable periods. DAH and DBH were mixed with 2 basal diets, because we(5) have found that chronic toxicity may be greatly affected by diet. The diets chosen were: (A), a ground, commercially available laboratory chow, and (B), our usual basal diet, containing in %: corn meal, 73; linseed oil cake meal, 10; alfalfa meal, 2; casein, 10; cod-liver oil, 3; bone ash, 1.5; and NaCl, 0.5. Substances under study were added with thorough mixing to basal diets to furnish following concentrations: 0, 0.0125, 0.025, 0.05, 0.1 and 0.2% of diet. One hundred fifteen male and 60 female rats were used, 5 animals to a cage. There were 10 males on the control and on each experimental diet except the one with 0.0125% in basal diet A. This group and all groups of females contained 5 animals each. Body weights, food intakes and general appearance were observed weekly for 200-216 days, then half of the males of each group were autopsied, and all females receiving Diet A. The remainder were continued with monthly weighings until approximately 500 days from start of feeding, and then autopsied. Gross appearance of tissues was noted, and organ weights were recorded for animals autopsied at 200 days. From all animals portions of heart, lungs, liver, kidney, intestine, stomach, thyroid, spleen, bladder and testis or ovary and uterus were fixed in formaldehyde. Paraffin sections stained with hemotoxyl and eosin were prepared for histological examination. Because our primary interest was on effects of continued ingestion, only a few animals were used in the negative study of acute oral toxicity. Each hydroquinone derivative was administered by stomach tube as 20% solution in ethyl alcohol to one rat at dosage levels of 500 and 1000 mg/kg, and as 30% solution in cottonseed oil to 5 rats at 1500 mg/kg level. Reactions were compared to those produced by hydroquinone as a 20% solution in ethyl alcohol. Rabbits and guinea pigs were studied for skin irritation. Ten per cent solutions of DAH and DBH in ethyl alcohol or cottonseed oil were prepared and 0.1 ml of each solution was placed on skin daily, except on week-end, for 2 weeks. Development of lesions and time of healing were noted. Studies of skin sensitization were made on guinea pigs by the

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method of Draize, Woodard and Calvery(6). For this test, suspensions of hydroquinones were made by dissolving 0.1 g of the crystals in 5 ml of ethyl alcohol, adding 0.1 ml of Tween 80 and diluting to 100 ml with 0.9% NaCl solution. Oettel(7) reported that daily oral administration of hydroquinone to black cats caused achromotrichia. To see if DAH and DBH had a similar action, each was mixed with ground rabbit chow and fed to black guinea pigs *ad lib.* 3 weeks. Concentration was 0.4% the first week, and 0.2% thereafter. No clearly discernible bleaching of hair occurred with either compound. Longer feeding was not possible because of unacceptability of food. A single black kitten was given DBH daily except week-ends for 2½ months. During final 6 weeks, the daily dose was 100 mg, in capsules. A partial browning (not graying) of hair occurred, probably attributable to aging, since the hair did not regain its former black color when DBH administration was discontinued.

Results. Body weights of experimental animals receiving DAH or DBH were, with one exception, similar to those of controls at end of 200 days. The exception was a significant decrease in rate of growth of female rats receiving 0.2% DBH in diet B. These animals weighed 221 ± 4.1 g at this time, compared to 250 ± 5.2 g for controls. The significance of this decreased growth is less apparent in view of the lack of effect on all other groups.

Red and white cell counts and hemoglobin concentrations of rats on diets containing 0.2% DAH or DBH did not differ significantly from values of control rats after 100 or 200 days on the diets. Normal hemoglobin values were found after 500 days.

Weights of organs (liver, spleen, kidneys, adrenals, heart, and ovaries or testes) were determined for animals autopsied after 200 days. No trend towards change in weight of any of these organs with increase in concentration was observed. The gross appearance of these tissues and those from animals autopsied after 500 days did not differ significantly from that of control animals. Microscopic examination of tissue sections revealed no lesions which could be attributed to the hydroquinone derivatives as fed in these concentrations to male and female rats.

DAH and DBH in amounts up to 1500 mg/kg by stomach tube produced no symptoms other than mild depression attributable to the ethyl alcohol or a loose stool produced by cottonseed oil. As opposed to this, 500 mg of hydroquinone/kg produced tremors lasting several hours, and 750 mg/kg killed 2 of 3 rats. The acute oral toxicity of derivatives was clearly less than that of unmodified hydroquinone, even when considered on an equimolar basis.

When DAH and DBH were dissolved in alcohol and applied to the skin, evaporation of alcohol left a visible, crystalline deposit which usually was not irritating. When dissolved in cottonseed oil, an erythema appeared in 24 hours, disappearing in the next 24 hours. Repeated applications of the oil solutions increased hyperemia, led to papular eruption followed by scab-formation over entire area. When treatment was stopped, lesions gradually disappeared, leaving a normal-looking skin after about a month.

Skin sensitization to these compounds was not found.

Discussion. If these compounds were to be used to stabilize a mixed feed ingredient such as alfalfa meal, it can be assumed that concentration of the compounds on the ingredient would never exceed 0.05% and maximum concentration in mixed feed would be 0.005%. Rats in this experiment receiving 40 times this concentration (0.2%) of hydroquinone derivatives for as long as 500 days were normal, as far as can be judged by available data. The only sign of toxicity noted was a reversible irritation of skin when oil solutions of derivatives were applied to this tissue. This would be a hazard to persons applying the material rather than to animals eating the alfalfa meal. Suitable protection of body and thorough washing with soap of exposed areas should decrease the chance of contact irritation.

In this study there was no indication that the basal diet modified toxicity.

Chickens appeared to be much more susceptible to the butyl compound than were rats. Neither species reacted adversely to the amyl compound.

Summary. Two hydroquinone derivatives, 2,5-di-*tert*-butylhydroquinone and 2,5-di-*tert*-amylhydroquinone, have been suggested as antioxidants to protect carotene of dehydrated alfalfa meal. Neither compound modified growth, appearance, organ weights or histological structure of rats eating diets containing as much as 0.2% of antioxidants for 500 days. No symptoms of intoxication developed in exploratory tests on rats when single doses of 1500 mg/kg of compounds were given by stomach tube. These materials did not sensitize the skin of guinea pigs but did produce a reversible lesion on rabbit and guinea pig skin when applied daily to the skin as 10% solutions in cottonseed oil.

Histopathological examination of tissues was made by Dr. Lelland J. Rather, Stanford Univ. School of Med.

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Effect of Cortisone on Survival of Morphine Treated Guinea Pigs under Decompression Hypoxia.* (25716)

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It is well known clinically that morphine constitutes a hazard in respiratory impairment. It is generally stated that this is due to depression of the respiratory center. Morphine is known to inhibit release of ACTH following exposure to stressors(1,2) by actions on some site(s) in the central nervous system. Although the purpose served by increase in production of adrenal steroids following such exposure is not clear, it seems possible that this action of morphine may contribute to some of its adverse effects. The effect of simulated altitude upon mortality of guinea pigs with and without concurrent administration of morphine was investigated. Morphine increased mortality under these circumstances. Administration of cortisone and ACTH reduced mortality.

Methods. Guinea pigs weighing 300-350 g of either sex were employed. All animals received 5 ml of saline intraperitoneally at start of observation. Animals receiving morphine were injected with 2.5 mg (or 5 mg) of morphine sulphate/100 g body weight 10 minutes earlier. Those which received cortisone were injected subcutaneously with 60 mg/kg of cortisone acetate twice daily on 3 preceding days and ½ hour prior to injection of morphine. This seemingly large dose of cortisone acetate has been used routinely for experiments with guinea pigs in our laboratory since this species is relatively resistant to its anti-anabolic action(3). ACTH was administered in single dose of 8 U of corticotrophin gel (Organon) subcutaneously and 8 U of corticotrophin powder (Armour) intraperitoneally, 30 minutes prior to morphine injection. When administered in multiple doses, 8 U of gel was injected subcutaneously twice daily for 3 days prior to experiments. On day of experiment, gel and powder were administered 30 minutes prior to morphine injection as indicated above. Two types of evacuation chambers were used, one employing jars with a single animal and the other vacuum ovens with 4 animals at a

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TABLE I. Mortality of Guinea Pigs after 6 Hours at 25,000 Feet Simulated Altitude.

Group	n	Died	%
Control	70	26	37
Morphine, 2.5 mg/100 g	123	62	50
" , 5 "	24	18	75
" , 2.5 " & cortisone	76	17	22
" , 5 " " "	24	7	29
" , 2.5 " & ACTH (S)*	13	7	54
" , 2.5 " & ACTH (M)*	43	8	19

* S = Single dose; M = Multiple dose.

time. Average air space/animal was approximately 3200 cc. Air pressure was maintained by vacuum pump. It was observed that mortality was greatly affected by air temperature, humidity and rate of admittance of air. To assure constancy in the latter, the same pump was used throughout experiment. Air seeped into the chamber in uniform manner. The experiment was carried out in a small room where temperature could be set to approximately 85°F. A vaporizer was kept in operation constantly to humidify the air. A simulated altitude of 25,000 feet was employed. This was achieved within 30 seconds. Duration of decompression was 6 hours.

Results. Mortality of 37% occurred in guinea pigs placed in evacuation chamber at 25,000 feet simulated altitude (Table I). This included all animals found dead at end of 6 hour period. It cannot be stated which animals died from sudden decompression and which died during ensuing period. However, deaths occurred from both causes. Among animals which had received morphine, mortality was 50% when dose was 2.5 mg/100 g and 75% when dose was 5 mg/100 g. Morphine-treated animals which had received cortisone as well exhibited marked reduction in mortality to 22% in those receiving a dose of 2.5 mg/100 g and 29% in those receiving 5 mg/100 g. Repeated priming with ACTH appeared to be even more effective in reducing mortality (19%).

Corticoids were determined in urine from survivors at completion of 6 hour decompression period. The hypoxic animals exhibited 65% increase in excretion of corticoids/mg creatinine over that of controls. Morphine-treated hypoxic animals exhibited 20% decrease in excretion over that of morphine-treated controls. However, in view of disturbance in cardio-renal hemodynamics which must be marked under such conditions, these findings cannot be considered reliable.

Discussion. Cortisone and ACTH reduced mortality of guinea pigs subjected to decompression hypoxia and morphine. These findings suggest increased requirement for adrenal cortical steroids under these conditions. Other conditions investigated (2,4) induced a large increase in corticoid excretion which was virtually abolished by morphine, but fatalities did not occur. It is possible therefore that corticoids played a particular role in hypoxic animals, for example by reducing oxygen requirements. This suggestion is not too unreasonable in view of the findings of Henneman and Bunker (5) that cortisone induced an increased excretion in lactic acid. Possibly cortisone increased availability and utilization of energy derived from glycolysis. This might be a fundamental purpose for activation of the hypothalamus, anterior pituitary, adrenal complex by stressors.

Summary. Mortality of 37% was induced in guinea pigs under decompression to a simulated altitude of 25,000 feet for 6 hours. Morphine sulfate increased mortality to 50%, and 75% if administered at dose of 2.5 mg or 5 mg/100 g body weight respectively. Prior treatment with cortisone for 3 days reduced mortality to 22% and 29% respectively. Multiple injections with ACTH reduced mortality to 19%.

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Mono-Substituted Vit. B_{12} Amides. II. Further Inhibition Study.*† (25717)

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Methylamide, ethylamide, and anilide of the monocarboxylic acid of vit. B_{12} were inactive as B_{12}-antagonist for B_{12}-requiring organisms(1); the highest concentration tested was 1 mμg/ml. In another study these amides in concentrations above 100 mμg/ml inhibited B_{12} utilization by *Ochromonas malhamensis*(2). Here we compare microbiological activity for 4 different B_{12}-requiring organisms of mono- and dibasic acid of B_{12}, the monoacid ethylamide, and a carbanilide derivative. Preparation of mono-amides has been reported(3,4); monoacid of ethylamide and the di-(ethylamide) were prepared by reaction of 1 or 2 moles of ethylamine with dibasic acid of B_{12}. Smith also prepared the carbanil derivative by linking it with one or both of the ribose hydroxyls. We extended previous study of monosubstituted amides because a clinical trial on an adult leukemia patient indicated antagonism against B_{12} storage in the liver.

Methods. The organisms used were *Lactobacillus leichmannii,* ATCC 7830, *Escherichia coli* 113-3, ATCC 11105, *Euglena gracilis,* Z strain, ATCC 12716, and *Ochromonas malhamensis,* ATCC 11532. *L. leichmannii* was grown on prepared dry mix from Difco Labs. B_{12} assays with these organisms have been described(5,6,7). The anilide, methylamide, ethylamide, monobasic acid, dibasic acid, ethylamide monoacid, di-(ethylamide), and carbanilide derivatives of vit. B_{12} obtained as powders, were dissolved in distilled water. Solutions were stored at 4°C with volatile preservative(8).

Results. Effect of substituted B_{12} amides and acids on growth of *O. malhamensis* and *E. coli* is summarized in Tables I and II. *O. malhamensis*: The methylamide, monobasic acid, and carbanilide promoted growth comparable to cyanocobalamin. The anilide permitted some growth, the greatest at 10^4 mμg/ml. The dibasic and ethylamide monoacids, and di-(ethylamide) did not support growth; they sharply inhibited B_{12} utilization above 10^2 mμg/ml. Inhibition by anilide was not as strong; it even supported some growth at 10^4 mμg/ml. *E. coli* 113-3: Methylamide and carbanilide permitted full growth; dibasic acid permitted growth above 10^3 mμg/ml. Anilide, ethylamide, monobasic acid, dibasic acid, ethylamide monoacid, and di-(ethylamide) antagonized B_{12} utilization above 10^2 mμg/ml. For *O. malhamensis* and *E. coli* appropriate B_{12} concentrations annulled inhibitions by these compounds indicating competitive type of inhibition. With *E. coli* grown in suboptimal concentrations of DL-methionine instead of B_{12}, as concentration of amides and acids were increased, full growth was attained; in absence of methio-

TABLE I. Effect of B_{12} Amides and Acids on Growth of *Ochromonas malhamensis.**†

Compound	Conc. (mμg/ml)	B_{12} (mμg/ml) 0	.01	.1	1
Control		.1	.78	2.8	3.5
Anilide	1	.24	.86	2.5	3.3
	10	.42	.94	2.3	3.2
	10^2	.46	.84	1.1	3.8
	10^3	.54	.78	.9	2.9
	10^4	.56	.8	.86	1.0
Methylamide	1	.9	1.0	2.3	3.2
Monobasic acid	10	2.6	3.1	3.7	3.5
Carbanilide	10^2	3.2	3.9	4.0	3.8
	10^3	3.3	3.6	3.8	3.7
	10^4	3.4	3.7	3.8	3.8
Ethylamide mono-	1	.16	.8	3.3	3.0
acid	10	.04	.6	3.5	2.7
Di-(ethylamide)	10^2	.02	.04	1.8	2.7
	10^3	.02	.02	.04	.8
	10^4	.02	.02	.04	.04
Ethylamide	1	.14	.8	3.4	3.9
Dibasic acid	10	.1	.7	3.0	3.6
	10^2	.12	.1	3.6	2.6
	10^3	.1	.06	.32	1.8
	10^4	.1	.1	.26	.2

* Data given were observed with first member of each group. Results with other members were similar.

† Growth expressed in optical density units, as measured with Welch Densichron equipped with a red-sensitive probe.

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† The authors thank Dr. E. Lester Smith of Glaxo Labs., England for substituted vit. B_{12} derivatives.

TABLE II. Effect of B_{12} Amides and Acids on Growth of *E. coli* 113-3.* Growth measured as in Table I.

Compound	Conc. (mμg/ml)	B_{12} (mμg/ml) 0	.1	1	10
Control		0	.42	.84	.96
Methylamide	1	.12	.66	.88	.94
Carbanilide	10	.66	.76	.88	.92
	10^2	.72	.74	.76	.88
	10^3	.74	.74	.76	.8
	10^4	.72	.76	.76	.78
Anilide	1	.0	.48	.88	.92
Ethylamide	10	.0	.54	.9	.92
Ethylamide mono-	10^2	.0	.14	.88	.92
acid	10^3	.0	.06	.38	.88
	10^4	.0	.06	.08	.44
Di-(ethylamide)	1	.06	.38	.94	1.0
	10	.06	.36	.96	.98
	10^2	.06	.4	.96	1.0
	10^3	.06	.2	.94	.96
	10^4	.04	.02	.76	.96
Dibasic acid	1	.1	.4	.94	1.0
	10	.1	.54	.98	1.0
	10^2	.2	.64	.6	.94
	10^3	.74	.68	.8	.96
	10^4	.84	.82	.86	.92
Monobasic acid	1	.0	.66	.92	.92
	10	.0	.12	.78	.96
	10^2	.0	.06	.18	.86
	10^3	.0	.06	.08	.16
	10^4	.0	.06	.06	.06

* Data given were observed with first member of each group. Results with other members were similar.

nine the amides permitted no growth (Table III).

All preparations were about as active for *E. gracilis* and *L. leichmannii* as was true B_{12}.

Discussion. Methylamide and anilide of B_{12} antagonize growth of *E. coli* 113-3 and *O. malhamensis*(2,9). In this study the anilide, but not the methylamide, inhibited. Inhibition of *O. malhamensis* by ethylamide confirms Ford(2). Previously(1) we had seen no inhibition because concentrations of the antagonists above 1 mμg/ml were not used. Growth promotion by methylamide does not agree with previous results(1). This compound stimulates both growth of *E. coli* and *O. malhamensis*. The fresh batch, reported here, may have been contaminated with small amounts of true B_{12}; this was not so with the previous preparation(1). To test this assumption, the preparation of methylamide sent by Smith, upon which previous observations(1) were based, was restudied. As before, no stimulation was noted.

The stimulation produced by monobasic acid for *O. malhamensis* without comparable stimulation for *E. coli* rules out contamination by true B_{12} (cyanocobalamin) or other cobalamins active for higher animals(10); perhaps *O. malhamensis* converts this derivative to true B_{12} or else uses B_{12} with such an altered cyanocorphinamide.

Methionine results with *E. coli* agree with our earlier ones(1). Suboptimal levels of methionine act synergistically with B_{12} amides and acids enhancing *E. coli* growth. Methionine supplies methyl groups which may permit B_{12}-antagonists to act as true B_{12} in syntheses requiring methyl group transfer (11). Hence the B_{12}-antagonists, at least in *E. coli* 113-3, act by inhibiting methyl group synthesis; if preformed methyl groups are supplied, the bacterium retains its ability to transfer these groups.

Assuming no B_{12} contamination, carbanilide activity for *O. malhamensis* and *E. coli* shows that both organisms can convert or utilize this compound as well as true B_{12}. The reason dibasic acid antagonizes B_{12} utilization for *O. malhamensis* and not for *E. coli* is obscure.

The activity noted here for various antagonists against B_{12}-requiring organisms appears

TABLE III. Effect of B_{12} Amides and Acids on *E. coli* 113-3, Grown on DL-methionine.*†

Compound	DL-methionine (μg/ml) 1	3	10
Control	.08	.14	.52
Anilide	.14	.26	.6
Ethylamide	.22	.42	.66
Monobasic acid	.26	.52	.7
	.44	.62	.78
	.42	.62	.9
Methylamide	.4	.52	.68
Dibasic acid	.7	.74	.84
Carbanilide	.8	.74	.84
	.78	.8	.86
	.8	.8	.88
Ethylamide monoacid	.12	.26	.54
Di-(ethylamide)	.14	.36	.64
	.18	.4	.66
	.16	.44	.66
	.18	.46	.68

* Data given were observed with first member of each group. Results with other members were similar.

† No growth without DL-methionine.

to reflect subtle differences in B_{12} metabolism. One might postulate that the greatest use of these compounds would be in clinical entities embodying highly deranged B_{12} metabolism, as in leukemia and liver disease(12,13,14).

Summary. The anilide, ethylamide, ethylamide monoacid, and di-(ethylamide) derivative of B_{12} inhibit cobalamin utilization by *O. malhamensis* and *E. coli.* The monobasic acid inhibits B_{12} utilization by *E. coli,* but not by *O. malhamensis*: the reverse is true for the dibasic acid. Methylamide and carbanilide derivatives are utilized as well as true B_{12}; all compounds are as active as true B_{12} for *E. gracilis* and *L. leichmannii.* In *E. coli,* suboptimal levels of methionine in presence of B_{12} amides and acids give increasing growth with increasing concentrations of B_{12} derivatives; neither methionine nor B_{12} acid and amide, when added alone, permit growth.

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Influence of ACTH upon Avian Species and Osteoporosis.* (25718)

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During previous investigations(1,2,3), osteoporosis was found in the culls of a flock of White Leghorn hens bred for heavy egg production. Endocrine interactions and role of pituitary, adrenals, and gonads in pathogenesis of this disorder are obscure. In the human, *species in which osteoporosis is common,* it is debatable whether exogenous ACTH is a causative agent or whether it only aggravates a pre-existing condition. Adrenal cortex increases in size and weight during reproduction in birds, due to hyperplasia and hypertrophy and occurs presumably through increased secretion of ACTH. In White Leghorn hens and roosters, the cortex represents 43% of weight of both adrenal glands(4). Bovine ACTH increases size of adrenal cortex, and prolongs survival of hypophysectomized birds; cortisone sustains the animal for indefinite period. Both ACTH and cortisone are powerful suppressors of bone growth in the chick(5). Pituitary - adrenocortical - gonadal hormone interactions have been demonstrated in birds(4,6,7) by the following experiments: (a) castration causes enlargement of adrenal cortex; (b) unilateral ovariectomy is followed by hypertrophy of opposite vestigial ovary if adrenals are intact but not if adrenal glands are also excised; (c) adrenal cortex may react to injections of small or large doses of estrogen or androgen in various ways, directly and indirectly through liberation or suppres-

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TABLE I. Reaction of Adrenal Glands, Ovaries, and Skeleton to ACTH.

No. of birds	Sex	Dose (mg lyophilized ACTH)	Days	Ovary wt, g/kilo	Adrenal wt, mg/kilo	Serum calcium	Blood sugar	Avg No. eggs/wk	Intramedullary bone†	Osteoporosis‡
7	♂		14		55.1 ± 5*	9.5 ± .5*	200. ± 21*		.0	1.5
7	♀		"	11.4 ± 2*	80.1 ± 3	20.5 ± 2.1	232. ± 28	6	1.	1.
4	♂	240	"		41.9 ± 5	8.8 ± .4	239.6 ± 22		.0	1.5
4	♀	"	"	10.1 ± 2	93.1 ± 3	34.5 ± 2.5	235.4 ± 19	4	.5	1.
2	♀	525	21	5.9 ± 1	91.1 ± 2	21.1 ± 1.9	230.1 ± 24	5	1.5	.7
2	♂	480	28		40.7 ± 3	10.6 ± 1.	208.1 ± 19			.0
2	♀	"	"	7.2 ± 2	101.2 ± 5	19.1 ± 1.	228. ± 18	5	1.5	.5
2	♂	770	42		58.1 ± 2	12.64 ± .6	200.5 ± 15		.0	.0
2	♀	"	"	3.4 ± 1	109.4 ± 5	54. ± 4.	239. ± 26	4	2.5	.2
2	♀	70	49	9.9 ± 2	81.5 ± 2	28.7 ± 2.1	221.4 ± 14	4	2.5	.2

* Mean ± stand. error. † Avg thickness of deposit in mm. ‡ Avg thickness of cortex in mm.

sion of ACTH by the pituitary, but in general estrogen increases and androgen decreases weight of adrenals; (d) enlargement of adrenal gland following castration is prevented by administration of androgens. Our object is to measure levels of plasma corticosterone and 17-OHCS (17-hydroxy-corticosteroid), weight of adrenal glands, and gonads, and to correlate such data with calcium and bone metabolism.

Materials and methods. Fifteen adult Black Minorca roosters and 19 White Leghorn hens, purchased in open market and reared on standard commercial feed, were treated with lyophilized ACTH, 40 mg/injection at intervals and for periods of 14-49 days. Observations were made on various constituents of serum including proteins by paper electrophoresis, gross and microradiography, routine microscopic and special histochemical preparations, by methods described previously(2,3). Adrenal glands and ovaries were weighed wet before fixation in formalin for histological sections. Heparinized blood was collected from 14 untreated birds and 10 birds treated with intraperitoneal or intramuscular injections of either lyophilized or oxycel processed ACTH for 1 week. Level of 17-OHCS in plasma was measured by method of Nelson and Samuels(8); corticosterone was measured by method of Silber, *et al.*(9).

Results. Control roosters. Weight of 2 adrenal glands/kilo of control rooster was only 55.1 ± 5 mg (Table I). Level of blood sugar was approximately 200 mg%. There was no intramedullary new bone. Level of corticosterone was more than twice that of 17-hydroxy-corticosteroid (Table II).

Control hens. Weight of adrenal glands/kilo of control hen was 80.1 ± 3 mg. White Leghorn hens weighing an average of 1.6 kilo

TABLE II. Levels of Corticosterone and 17-Hydroxycorticosterone in Normal and ACTH-Treated Birds.

No. birds	Sex	Dose ACTH	Corticosterone Range in γ/100 cc plasma	17-OHCS Range in γ/100 cc plasma
7	♂		6.5 ± 2	2. ± 1
7	♀		8. ± 3	2.5 ± 1
5	♂	400 IU	12 ± 5	"
5	♀	"	15 ± 4	"

had adrenals weighing 60% more than Black Minorca roosters weighing an average of 3.5 kg. Level of blood sugar was as high as 232 mg %. Plasma level of both corticosterone and 17-hydroxy-corticosterone was also slightly higher in the laying hen than in the rooster. Deposits of intramedullary bone in the tibia varied from 0.5 to 2.5 mm in thickness from one phase to another in the egg-laying cycle.

Roosters injected with ACTH. Injections of mammalian ACTH produced slight increase in weight of avian adrenal gland. Level of corticosterone in plasma, however, increased approximately 2-fold (in both sexes); level of 17-hydroxy-corticosterone changed little if at all. Changes in chemical composition of other constituents of blood were hardly appreciable compared to effects observed previously in estrogen-treated birds(2). No change was found in serum total lipid, phospholipid, total nitrogen, albumin, globulin, A/G ratio, total phosphorus, inorganic phosphorus, or alkaline phosphatase. Blood sugar was elevated very slightly if at all. At conclusion of experiment in birds treated 6 weeks, there was a slight hypercalcemia. Bones showed slight, if any, thinning of cortex, and no intramedullary osteogenesis. There was no demonstrable evidence of osteoporosis.

Hens injected with ACTH. Adrenal glands in hens were enlarged and increased in weight. Level of corticosterone in plasma increased significantly while 17-OHCS remained unchanged. Ovaries became progressively smaller at 2, 4, and 6 weeks after treatment. The reduction was apparently proportional to dose of ACTH and duration of treatment. In hens previously in heavy lay, egg production was gradually reduced from 6-7 to 4 eggs/week. Level of serum calcium in hens observed after 2 and 4 weeks treatment varied (as in control hens) from 10 to 35 mg%. In 2 hens observed after 6 weeks, total calcium was even higher. ACTH did not inhibit synthesis of phosphoprotein by the liver, but some calcium added to serum may not have been protein bound. Paper electrophoresis showed X_1-X_2 bands but they were not as dense as in control hens. Serum lipid and phospholipid and other components normally found in blood of laying hens were the same in treated birds and control birds. Level of blood sugar was not elevated. The cortex of long bones was thinner in hens than in roosters and showed increased number and size of vascular spaces; bone resorption appeared to occur without osteoclasts, and osteoporosis was extreme in hens treated with ACTH for 6 weeks. The marrow cavities were filled, however, with poorly calcified or uncalcified bone. The intramedullary deposit was not detached from the cortex as occurs in hyperparathyroidism or Vit. D and calcium deficient hens(3).

Discussion. The adrenal vein blood of the capon has been observed by Phillips and Jones (11) to contain 312 γ of corticosterone and 3 γ % of hydrocortisone. Our analyses of peripheral blood plasma of intact roosters and laying hens revealed 3 to 6 times higher levels of corticosterone than 17-OHCS. However, we found approximately the same concentration of 17-OHCS, only 2.5 γ %, in systemic blood as Phillips and Jones measured in adrenal vein blood. Using a small dose of ACTH, they were not able to stimulate secretion of corticosterone in adrenal vein blood; using 400 I.U. of ACTH, ACTH did raise the level of corticosterone in plasma, as well as increase size and weight of adrenal glands. The effect on the skeleton of pharmacodynamic doses of ACTH was to accentuate osteoporosis to which the White Leghorn female, but not the male, is predisposed. Resistance of rooster to osteoporosis may be related to metabolism of (precursors of) androgens; it is accordingly necessary to measure the output of these and related substances in blood or excreta.

Body weight. Doses of ACTH in above experiments produced gradual loss of body weight in hens in 6 weeks. Roosters were less affected in this respect. Loss of body weight is generally attributed to widespread catabolism of the labile fraction of body protein, especially muscle, similar to that which occurs in inanition. The bones of the hen were hard but became lighter and more brittle after ACTH. The osteons on the endosteal side of cortical bone were removed and replaced by large vascular spaces, fibrous tissue, and spongy bone. This occurred without formation of osteoclasts.

Level of the blood sugar normally varies from 161 to 280 mg % in birds. The range in hens and roosters treated with ACTH was 200.5 to 239.6, indicating no effect on blood sugar. In this respect, birds differ from mammals in which ACTH and adrenal corticoids have diabetogenic effects(10). Prolactin may elicit this response in oviparous animals.

The large dose of ACTH used in the foregoing experiments diminished ovarian weight and egg production steadily for 6 weeks. These changes could be attributed to suppression of production of gonadotropin by the anterior pituitary or secretion of estrogen by the ovary, or both, by corticoid hormones.

Adrenal cortex. The literature reviewed by Sturkie(10), reveals that ACTH extracted from the pituitary of a lower order can produce a response from an animal of a higher order, as well as *vice versa.* Riddle and his associates(12-14) observed a 40% enlargement of adrenal cortex and a 20% increase in level of blood sugar in doves and pigeons during reproductive cycle. Because egg production ceased in adrenalectomized birds, Riddle, *et al.*(14) observed that the adrenal cortex is essential for reproduction. It is reasonable to suppose that while mammalian ACTH affects avian adrenal cortex as evidenced by depletion of adrenal ascorbic acid (15), and(2) production of corticosterone (as shown above), avian ACTH might produce a quantitatively greater response than injections of heterogenous hormone.

Calcium protein complexes. A hypercalcemia which occurs in the laying hen is associated with formation of a calcium phosphoproteinate complex, and deposition of egg yolk, and this occurs under control of estrogen(1). In small doses the process is not altered by ACTH and adrenal cortical hormone. A different form of hypercalcemia, not associated with formation of phosphoprotein (as shown by chromatographs of their serum) occurs following prolonged treatment with large doses of ACTH. This hypercalcemia was relatively slight and could have been due to suppression of new bone formation by excess production of corticosterone. These observations require further investigation by studies of urinary and fecal excretion of calcium.

Intramedullary osteogenesis, like a secondary sex characteristic, is under control of estrogen and its function is to store and turn over a supply of calcium for calcification of the egg shell(3). Osteoporosis is a disorder consisting of increased porosity and decreased thickness of the cortex; the pathogenesis is not known.

Injections of ACTH for 4 weeks caused retention of intramedullary bone. At beginning of period of treatment while the hen was in heavy lay, overproduction of adrenal corticoids did not prevent synthesis of the proteinaceous matrix of intramedullary bone. Later, when total body weight was reduced and the cortex showed marked osteoporosis, egg production came to a halt and this relieved the skeleton of the demand for calcium for egg shells. Treatment with cortisone, as will be described(1), similarly produced retention of intramedullary bone and severe osteoporosis in laying hens. Storey investigated the action of ACTH, reviewed the literature on effect of adrenal steroids on bone in mammals and concluded that ACTH suppressed growth but did not produce osteoporosis in young rabbits(16). The circumstances in an adult animal are entirely different especially in the modern laying hen, now apparently under great stress. The modern laying hen in heavy lay has been observed to come off her legs as a result of a disorder known as "Cage Layer Fatigue"(17). CLF in birds, shortly after onset of lay, is associated with "decalcification of bones" without any change in serum calcium, phosphorus, or alkaline phosphatase(18). After a long period of laying when the pullet becomes an adult, the bones of a bird with CLF may show the characteristics of osteoporosis as described above.

Summary. Osteoporosis, occurring spontaneously in White Leghorn hens, was aggravated by injections of mammalian ACTH; the disorder did not develop in the male. It is suggested that there may be a causal relationship between ACTH, osteoporosis and the modern poultry disorder known as "Cage Layer Fatigue."

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Erythropoietic Activity of Proteolytic Enzymes. (25719)

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During some studies on effect of certain enzymes on activity of erythropoietin preparations, we found that samples of papain and bromelin could stimulate erythropoiesis. Consequently, a number of other proteolytic enzymes were assayed.

Methods and materials. A modification of the methods of Fried *et al.*(1) and Rambach *et al.*(2) was used to measure erythropoiesis. Each sample was injected subcutaneously daily for 4 days into 6 female Holtzman rats. Food was withheld throughout experiment but water was given *ad lib.* On fourth day, 0.5 μc of Fe^{59}, as ferric citrate, was injected into the tail vein. Twenty-four hours later amount of radioactive iron in an aliquot of whole blood was measured. In calculating % of Fe^{59} uptake by total red cell mass, it was assumed that blood volume was 5.18% of body weight at time of sacrifice. *Enzymes* were obtained from commercial companies (Table I). Sialic acid was determined by direct Ehrlich reaction described by Werner and Odin(3).

TABLE I. Erythropoietic Activity of Proteolytic Enzymes.

Enzyme	Purity	Source	Dose, mg	Fe^{59} uptake, avg %
Pepsin	Crystalline	Worthington	.5	7.4 ± .9
Trypsin	"	"	.1	11.1 ± 1.6
Chymotrypsin	"	"	.1	11.2 ± .2
B. subtilis proteinase	Crude	Novo	.5	9.2 ± 1.1
Bromelin	"	Takamine	.5	13.6 ± 1.1
Ficin	"	Lilly	.5	9.1 ± 1.4
Control				10.3 ± .9
Papain	Crude extract	Penick	5.0	14.3 ± 1.2
Control				7.8 ± 1.0
Papain	Partially purified	Lilly	.2	13.1 ± 1.3
Control				10.1 ± 1.5
Papain	Crystalline	Worthington	.1	13.4 ± 1.5
Control				10.3 ± .9

Results. Erythropoietic activity was detected in crude preparation of bromelin and in crude, partially purified, and crystalline preparations of papain. Other proteolytic enzymes showed no activity. The data are summarized in Table I.

Crystalline papain contained 1.1% of sialic acid.

Discussion. Our results raise the following questions: (1) Is erythropoietic activity of papain related to its proteolytic activity or to a specific structure of the enzyme molecule? (2) Is sialic acid an impurity in crystalline papain preparations or is it part of the enzyme molecule? These require further investigation.

Summary. Crystalline papain and crude bromelin preparations stimulate erythropoiesis, whereas other proteolytic enzymes are ineffective. Sialic acid, measured by direct Ehrlich reaction, has been detected in crystalline papain preparations.

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Thyroid Hormone Binding Proteins in Human Serum Characterized by Immuno-Electrophoresis and Auto-Radiographic Tracings.* (25720)

J. CLAUSEN AND T. MUNKNER (Introduced by G. Asboe-Hansen)

There is no general agreement on the nature of linkage of thyroxin and triiodothyronine to human serum proteins(1). Identification, isolation and investigation of these binding proteins are essential to understanding the transport of thyroid hormone and may involve a better knowledge of metabolism of the hormone. By a combination of immuno-electrophoresis of human sera incubated with radio-thyroxin and radio-triiodothyronine and autoradiographic technic it has been possible to identify exactly the thyroxin- and triiodothyronine-binding proteins as the same 3 lipoproteins. This combination of immuno-electrophoresis with tracer technic seems to be the method for identifying and estimating linkage of organic and inorganic compounds to serum proteins.

Methods. Immuno-electrophoresis(2) was performed as described by Scheidegger(3) at pH 8.6 and stained for serum proteins. Lipoproteins were demonstrated with Oil red O (4). Ten to 20 μl of human serum were incubated with corresponding volume of a radio-L-thyroxine or radio-L-triiodothyronine solution in 50% propylene-glycol (Abbott Laboratories). Activity was 2-3 μc/sample and by incubation about 0.3 10^{-9} M thyroxin or 0.2 10^{-9} M triiodothyronine were added to original hormone content of serum. After immuno-electrophoresis the slides were placed in direct contact with Kodak X-ray film or Kodak autoradiographic film for a week and then developed.

Results. Fig. 1 shows immuno-electrophoresis of normal serum incubated with thyroxine (upper half) and triiodothyronine (lower half) and stained conventionally for serum protein precipitation bows with amido-black. Fig. 2 shows corresponding autoradiographs. Only 3 different precipitation bows contain radioactivity. From their mobility these 3 thyroxine- and triiodothyronine-binding proteins must be identified with the 3 known lipoproteins in normal human serum, *i.e.,* alpha-2-lipoprotein (the slow moving lipoprotein fraction), alpha-1-lipoprotein (the

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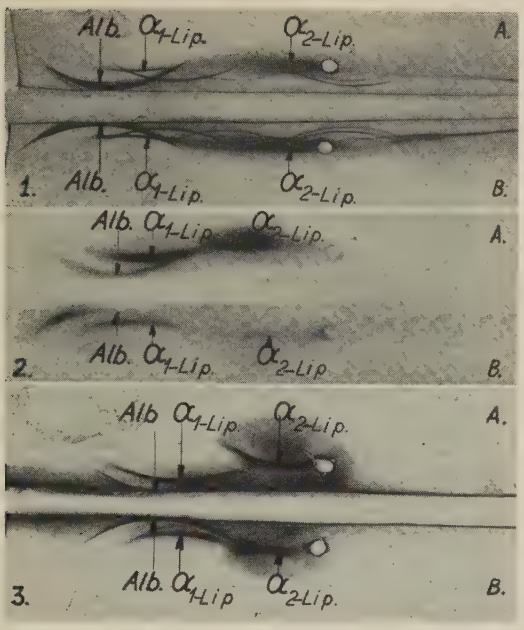

FIG. 1. Photo of immuno-electrophoresis colored conventionally for serum protein precipitation bows with amidoblack. A. Immuno-electrophoresis of normal human serum incubated with thyroxine. B. *Idem* with triiodothyronine.

FIG. 2. Photo of same tracings as in Fig. 1 developed as autoradiogram.

FIG. 3. Photo of immuno-electrophoresis of same sera as in Fig. 1 colored for lipoproteins with Oil red O.

faster moving lipoprotein, the mobility of which may vary to some extent and range from prealbumin area to the cathodical part of the alpha-1-area, depending on age of serum), and lipalbumin. Fig. 3 shows immuno-electrophoretical tracings of sera stained for lipoproteins with Oil red O, autoradiographic blackening exactly corresponding with the 3 lipoproteins.

Discussion. Previous studies concerning the possible existence in serum of a specific thyroxine- or triiodothyronine-binding protein have been confusing, because conventional methods for separating serum proteins do not correlate, giving fractions with different properties. *Salting out procedures* give no sharp zone of precipitation for identification of such a protein. *Moving-boundary electrophoresis* (5), too, reveals a varying iodination of the different protein fractions. Robbins & Rall (6) and Horst & Rösler(7) using *conventional paper-electrophoresis* found that thyroxine was bound to a protein fraction in the area between alpha-1 and alpha-2 globulins and to a smaller extent to a fraction in the albumin area. *Acid electrophoresis and ultracentrifugation* have suggested thyroxine-binding protein to be an alpha-2- glycoprotein with a sedimentation rate $S_{20,w}$ of 3.3(8) and (9). *Cohn fractionation*(10,11) seems to indicate that the protein is localized to fraction IV, V and possibly to VI. Fractions IV and V mainly contain albumin, alpha- and beta-globulins(12). Finally Rich and Bearn (13) using *starch gel electrophoresis* found a high specific thyroxine binding capacity in the prealbumin area.

In immuno-electrophoresis only the antigen-antibody precipitate corresponding to each protein fraction in serum is left, whereas any excess of antigen or antibody is removed by repeated washings with saline. Hence only protein linked thyroxine and triiodothyronine involved in the precipitate will remain on the slide, while all free hormone, all thyroxine or triiodothyronine which was only loosely bound to the precipitate, and any complexes between hormone and nonreacting proteins will not be retained in the immuno-electrophoresis. We therefore conclude that only the 3 lipoproteins in human serum have the capacity to bind thyroid hormone in serum. We could not confirm a specific and very firm binding between any carbohydrate-containing alpha-serum protein (for example Schultze's alpha-2-macroglobulin or the alpha-1-glycoprotein(14)). The results obtained by other authors using more conventional fractionation methods can be explained by immuno-electrophoretical findings in prealbumin, albumin, and alpha areas. Immuno-electrophoresis can unveil 3 different well known serum proteins in the alpha-1 area (*i.e.*, alpha-1-lipoprotein, alpha-1-glycoprotein and alpha-1-seromucoid) with almost the same mobility, whereas these proteins cannot be separated by paper electrophoresis. Next, mobility of alpha-1-lipoprotein is variable (15) depending on how long the serum has been stored. Fresh alpha-1-lipoprotein shows an alpha-1-mobility, whereas serum stored for some time appears with some splitting of lipoprotein, giving rise to a new precipitation

bow with prealbumin mobility(15), so that the sum of the amount of alpha-1-lipoprotein and lipo-prealbumin remains constant. The complex of alpha-1-lipoprotein and radio-thyroxine or radio-triiodothyronine on auto-radiograph may therefore show some variability as a function of storing and possibly, by chemically induced splitting by addition of the labelled hormone. Also the lipoprotein with alpha-2-mobility seems to have a somewhat variable position depending on amount of lipo- and normal serum proteins(15). Thus on the basis of immuno-electrophoretical tracings within the lipoprotein system and our investigation of binding properties, the confusing results which have been obtained concerning thyroxine binding proteins in human serum are understandable. The reason some workers have found a very high thyroxine binding capacity to the tryptophan rich prealbumin of Schultze(16), is being investigated, as our antisera have a very low titer against this protein.

Summary. Immuno-electrophoresis of normal human serum incubated with radio-thyroxin and radio-triiodothyronine combined with auto-radiography and special staining for lipoproteins with Oil red O showed that the albumin (identical with lipalbumin), alpha-1-lipoprotein and alpha-2-lipoprotein have the capacity to bind thyroxin and triiodothyronine.

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Effect of *Pseudomonas* on Lactate Production by Mouse Monocytes.* (25721)

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Recent data obtained in this laboratory indicate that succinoxidase activity of cells obtained from mouse peritoneal exudates, as well as of homogenates of liver and spleen, was inhibited in presence of heat-killed *Pseudomonas aeruginosa*(1). However, since metabolism of mouse monocytes appears to be mainly glycolytic, this study was undertaken to determine in what way, if any, *P. aeruginosa* might affect lactate production by these cells.

Method. Warm mineral oil (0.5 ml/mouse) was used to induce exudates in 10 week old female mice (Webster strain), 18 hours before harvesting of cells from peritoneal cavities with chilled 0.9% sodium chloride containing 0.1% heparin. Pooled monocytes were washed once with saline by centrifuging for 10 minutes at 500 x G. In experiments with

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infected monocytes, washed *Pseudomonas* (10^8) were administered intraperitoneally one hour before autopsy. Extracellular organisms were killed by incorporating polymyxin B (500 units/ml) in heparinized saline. Where indicated, exudates were obtained from mice immunized with suspension of dead *Pseudomonas* as previously reported(1). Most experiments employed intact cells, but where disrupted cells were required, homogenates of equivalent numbers of cells were prepared by sonication for 15 minutes in 9 KC Raytheon sonic oscillator. Counts with a Spencer hemacytometer indicated that 1 ml contained approximately 5 x 10^8 packed cells. All glycolysis experiments were run aerobically in heparinized phosphate buffer (0.1 M), pH 7.4, for 90 minutes. Lactic acid was determined by method of Barker and Summerson (2) and reported as amount/ml obtained from 20 μmoles of sugar/2 ml of a 5 x 10^7 cell suspension. Manometric experiments were run in conventional manner(3). Liver and spleen homogenates were prepared by homogenizing tissues in phosphate buffer, pH 7.4, for 60 seconds in Waring blendor.

Results. A survey of monocyte glycolysis, using lactate production as indicator, showed that only glucose and fructose were metabolized (Table I). Glucosamine, glucuronate, gluconate, methyl glucose, galactose, maltose, sucrose, fructose diphosphate, lactate, ribose, sorbose, l-xylose, mannose, arabinose, d-xylose, l-fucose, galacturonate, starch, dextrin, salicin, chondroitin sulfate, trehalose, raffinose, cellobiose, melezitose, melibiose, l-rhamnose, ascorbate, and glycogen were not utilized.

Since permeability may be a factor in preventing some sugars from being converted to lactate, monocytes were disrupted by sonic methods, and substrates related to glucose were tested with cell-free extracts. Nevertheless, substrates such as methyl glucose, glucosamine, glucuronate, galactose, maltose, and galacturonate were either not metabolized or were converted to substances other than lactate. Lactate production from glucose and fructose was not adversely affected by sonic treatment of the monocytes since cell-free preparations yielded 600 μg of lactate per ml with either sugar. This value compares well with 690 μg/ml obtained with intact cells. It should be pointed out that lactate production from glucose and fructose was almost always identical.

TABLE I. Lactic Acid Production by Monocytes from Normal and Immunized Mice.

Substrate	μg lactate/ml N*	I*
-(Endogenous)	160	158
Glucose	690	690
Fructose	680	685

* Monocytes from normal (N) and immunized (I) mice.

TABLE II. Lactic Acid Production by Infected* and Non-Infected Cells.

Substrate	μg lactate/ml Non-infected	Infected*
-(Endogenous)	71	150
Glucose	250	605
Net lactate	179	455

* Living *Pseudomonas* inj. intraper. into mice 90 min. prior to cell harvest.

Measurements of lactate production with infected and non-infected monocytes indicated greater acid production by infected cells. The results are seen in Table II. Infected cells yielded at least twice as much lactate as did uninfected cells whether supplemented with glucose or not. Net lactate production from glucose was 455 μg/ml for infected cells *vs.* 179 μg/ml for uninfected cells. Manometric studies suggested that the lactate produced with infected monocytes was solely monocytic in origin, since no intracellular microbial oxidation of either glucose or gluconate could be demonstrated. This assumption was supported by the fact that no net oxygen consumption could be noted using uninfected monocytes supplemented with glucose or gluconate.

Since previous experiments indicated that the succinoxidase activity of monocytes was susceptible to inhibition by bacteria and bacterial fractions, several different preparations of *P. aeruginosa* were added to monocytes to determine their effect on glycolysis. Heat-killed *Pseudomonas,* cell-free sonic extracts, and particles obtained by centrifuging sonic extracts of *Pseudomonas* were added to uninfected monocytes *in vitro.* Examination of

TABLE III. Lactic Acid Production by Monocytes Treated *In Vitro* with Intact and Fractionated *P. aeruginosa.*

	μg lactate/ml			
		*Pseudomonas**		
Substrate	—	Intact bacteria	Supernatant	Particles
(Endogenous)	40	39	48	43
Glucose	590	600	590	690
Fructose	585	590	585	595

* Heat-killed intact organisms, supernatant of heat-inactivated sonicated *Pseudomonas*, or unheated particles from sonicated bacteria were added to give a concentration of 2.5 mg N/ml.

Table III indicates that 2.5 mg N/ml of each microbial preparation had no inhibitory effect on lactate production by monocytes. Similar results were obtained when microbial extracts were added to either spleen or liver homogenates.

Discussion. The data indicate that mouse monocytes exhibit a highly selective substrate specificity. Such substrates as methyl glucose, glucosamine, and maltose are closely related to glucose and yet were not metabolized to lactate. Permeability apparently is not a factor in substrate specificity. It has been established that polymorphonuclear leukocytes store sugars in the form of glycogen and that lactate production proceeds endogenously from this substance(4). However, when glycogen was added as substrate to monocytes, no lactate was detected. Previous immunization of donor mice with *Pseudomonas* did not affect lactic acid production by monocytes.

Kun and Miller(5) injected Salmonella endotoxin intravenously in rabbits. Blood lactic acid increased, but succinic dehydrogenase system of muscle and liver was inhibited. Although their experiments differed from ours, they obtained similar results. Sbarra and Karnovsky(6) have also noted increased lactate production by polymorphonuclear leukocytes when phagocytosis of polystyrene particles was allowed to proceed *in vitro* in presence of serum. However, in our *in vitro* studies when bacteria were added to monocytes in absence of serum no change in lactic acid production occurred.

Since similar studies had indicated that the succinoxidase activity of monocytes and of spleen and liver homogenates was inhibited by small concentrations of both intact and disrupted *Pseudomonas,* it was interesting to note that these preparations had no effect on lactate production. The results suggest that glycolysis is more refractory to microbial inhibition than is succinoxidase activity.

Summary. Mouse monocytes, induced by intraperitoneal injection of mineral oil, produced lactate from only glucose and fructose. No net lactate production was noted from 29 other sugars. *In vivo* infection of monocytes resulted in at least a 2-fold increase in lactate production. However, no effect was noted when either heat-killed *Pseudomonas aeruginosa* or sonic fractions thereof were added to monocytes *in vitro.* No significant differences in lactate production were noted between cells obtained from immunized and non-immunized animals.

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Influence of Avocados on Serum Cholesterol. (25722)

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Serum cholesterol values are lower when dietary fats are of vegetable rather than of animal origin. It is generally agreed that while degree of unsaturation of lipid is important, it is not necessarily the sole factor involved(1). Because of its high lipid content (5 to 25%) and palatable and convenient form, it was decided to use the avocado for partial replacement of mixed dietary fat with a fat of vegetable origin. Although few analyses have been made of the composition of avocado lipids, some representative values are shown in Table I. Total lipid content and possibly its composition vary with variety of avocado and degree of ripeness.

Materials and methods. Sixteen white male patients aged 27 to 72 years in Veterans Admin. Hospital, Coral Gables, Fla., took part in this study. Five patients were on salt restricted diets in which daily intake varied between 5 to 10 g. Three diabetic patients were given prescribed diets with respect to calories, fats, carbohydrates and protein, maintained throughout the study. All others ate regular hospital diet. Venous blood samples were drawn from all patients twice a week before breakfast. Total, ester and free cholesterol values were measured in serum by Sperry and Webb method(3) and phospholipids by the method of Zilversmit(4). Fat and calories were estimated from food tray by the dietician and diet lists prepared each day. All supplemental feedings were also recorded. Total calories and weight of fat were calculated from standard charts or from chemical analysis as described. The Fuerte variety of avocado was used from Feb. to May 1959 and the Hass variety from June to Sept. 1959.* To estimate total lipids of avocados, aliquot of pulp was extracted continuously with alcohol-ether mixture and total lipids weighed after solvent removal. Analyses of edible pulp were made in Jan., Feb., March and August and average values for total extractable lipids were respectively 21.8, 22.8, 22.9 and 20.6%. The edible portion of avocados averaged 200 g. Since an estimated 2 to 4% of total lipids are nonsaponifiable and presumably not assimilated, an average of 20% lipid content was used in calculations. With 200 g of edible material the average fruit contained 40 g of lipid. During control period, *i.e.*, 8 to 56 days, all subjects consumed regular diets without avocados. When reasonably constant blood values were obtained, from 0.5 to 1.5 avocados/day were incorporated into diet as shown in Table III. Animal fat equivalent to half or more of the avocado lipid was removed from the basal diet of 17 subjects. In subject 10, however, the avocado was added to his unchanged basal diet. Daily fat consumption before and during avocado feeding period is shown in Table III. Blood values and diet calculations were continued for duration of avocado feeding period.

Results. A summary of average blood values of all subjects is presented in serial order in Table II. Measurements made during control period are compared

TABLE I. Lipid Composition of Avocado Oil.

Source	Calif. & Fla.	Calif.	
Variety		Hass	Fuerte
Saturated fatty acids (% of total fatty acids)			
Myristic	.1		
Palmitic	7.2	22.1	14.1
Stearic	.6	.7	.2
Arachidic	trace		
Unsaturated fatty acids (% of total fatty acids)			
Palmitoleic		11.0	5.5
Oleic	80.9	51.9	70.7
Linoleic	11.2	14.3	9.3
Non-saponifiable (% of total lipid)	1.6		2–4
Iodine No.	94.4		
Reference	(2)	*	*

* These data were kindly supplied by Judd C. Nevenzel, Univ. of Calif., Los Angeles.

* All avocados used were supplied by Calavo Growers of Calif.

TABLE II. Serum Cholesterol and Phospholipids Values in 16 Patients.

Control period				Avocado feeding period							
Total chol.	Free chol.	Chol. esters	Phospho-lipids	Total chol.	P value	Free chol.	P value	Chol. esters	P value	Phospho-lipids	P value
367.0	97.0	270.0	14.6	236.7	<.01	64.7	<.01	172.0	<.01	11.5	<.01
296.0	92.0	205.0	11.6	169.0	"	48.0	"	121.4	"	8.0	"
267.0	71.5	195.5	16.2	198.6	"	54.2	"	144.1	"	10.9	N S
249.0	63.0	185.8	10.6	179.8	"	53.0	"	126.8	"	8.8	<.01
215.6	60.6	155.0	11.0	171.6	<.02	52.3	N S	119.2	<.02	8.4	<.02
270.6	69.3	201.3	12.8	196.0	<.01	53.1	<.01	142.9	<.01	11.2	"
331.0	90.8	240.2	16.7	302.3	"	81.5	"	221.0	N S	14.3	<.01
240.0	62.3	177.7	12.7	203.0	"	67.0	N S	134.0	<.01	10.0	N S
290.3	78.3	212.0	12.6	258.0	N S	69.0	<.04	189.5	N S	10.7	<.04
300.0	94.6	206.0	14.2	301.0	"	83.4	N S	217.8	"	10.8	N S
259.0	72.2	186.8	11.1	235.5	"	68.4	"	167.1	.01	11.5	"
305.0	84.6	220.8	13.3	271.8	"	73.8	.01	198.0	N S	12.9	"
552.0	234.2	317.8	26.2	547.2	"	217.8	N S	329.0	"	27.7	"
271.0	67.4	203.4	12.2	253.1	"	62.0	"	190.1	"	11.3	"
233.0	81.8	151.4	15.6	212.0	"	86.3	"	125.7	"	10.3	"
299.0	85.6	213.6	12.4	254.0	"	75.5	"	179.0	"	12.5	"

Cholesterol expressed in mg/100 ml and phospholipids as mg lipid P/100 mg.
All data are arranged in serial order of patients.

with those taken during avocado feeding period. Percentage change and "P" value by Wilcoxon Rank test(5) are shown. Of 16 subjects, 8 gave decreases in total serum cholesterol from 8.7 to 42.8%, which were statistically significant. Although both free and ester forms decreased significantly, changes in esters generally exceeded those in the free form. Serum phospholipids decreased at the same time as did cholesterol values. In no subject did cholesterol or phospholipid concentrations rise significantly during avocado feeding period. Such increase failed to occur in spite of appreciable increases in fat and calorie intake in several patients. Average values for daily fat and total calorie consumption and body weight during control and avocado feeding periods are given in Table III.

In 2 patients (1st and 6th) at end of avocado feeding period, a second control period was instituted, followed by second avocado feeding. The first was particularly interesting, because total fat intake during first control and avocado periods was low but was considerably higher during both second control and avocado periods. In both patients despite differences in fat consumption and calorie intake between first and second se-

TABLE III. Dietary Values and Body Weights.

Control period				Avocado feeding period				
Days	Fat/day	Calories/day	Body wt	Days	Avocado/day	Fat/day	Calories/day	Body wt
22	57	2307	147	34	1.5	83	2577	143
21	41	1179	147	13	.5	72	1550	140
56	70	1582	165	31	1.5	76	1657	161
25	50	1237	156	36	1.0–1.5	59	1363	154
13	94	2271		25	1.5	103	2161	
37	46	1251	143	41	1.5	98	1833	145
14	44	1654	151	72	1.5	115	2706	152
20	25	1027	183	9	1.5	86	1666	186
8	71	1629	150	33	.5	71	1629	150
12	85	2029	154	21	1.0	154	2965	154
17	60	1228	170	50	.75	60	1228	162
14			132	85	1.5	105	2052	133
12	71	1834	139	76	1.5	102	2336	140
8			131	25	1.5	105	1972	129
19	27	838	160	39	.5	38	891	154
14	50	1200	130	20	.5	50	1200	127

All data are arranged in serial order of patients.

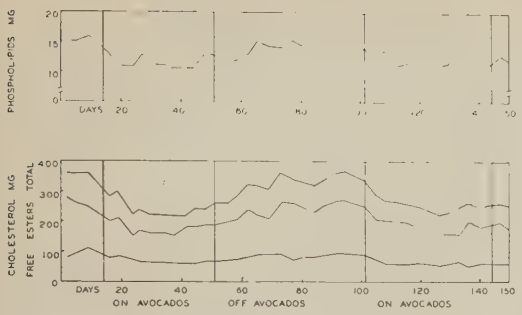

FIG. 1. Serum cholesterol and phospholipid values in a single subject.

ries, decreases in total cholesterol values were similar.

To illustrate time sequence in feeding trials, total, free and ester cholesterol, and phospholipid values obtained from one subject were graphed in Fig. 1. Total serum cholesterol and phospholipid values began to fall in a week after inclusion of avocados in the diet. Changes in ester form were far more striking than those in free cholesterol.

Discussion. Partial substitution of avocado lipid for mixed dietary lipids produced a significant decrease in serum total cholesterol values in 8 subjects. An obvious explanation lies in the unsaturated, fatty acid content of avocado fat which has an Iodine No. of 94 (Table I). Another possibility concerns the 1 to 4% unsaponifiable fraction of avocado lipid which appears to be a sitosterol in nature. When 10 g or more of sitosterol were fed daily a decrease in serum cholesterol was observed(6). One avocado contains about 40 g of lipid. Assuming an average nonsaponifiable fraction of 2%, a single fruit would have about 0.8% g of a sitosterol. Although daily consumption of sitosterol alone may have been ineffective, its combination with unsaturated avocado may have increased its efficacy.

Average daily fat and calorie consumption in general increased during avocado feeding period, yet body weights did not increase as expected (Table III) and in some subjects declined from 1.5 to 4.8%. Small changes in body weight, however, were not correlated with changes in serum cholesterol. This apparent discrepancy between increased fat consumption and changes in body weight is under investigation.

In 8 subjects, decreases in serum cholesterol values were not statistically significant. While there is no complete explanation for lack of response, there are certain differences between the group responding and that not responding. Of those showing no real change, 3 were mild diabetics and another had hypercholesterolemia of unknown origin. A fifth subject not responding was treated differently from all others in that the avocado was added to his diet without removing existing fat.

Summary. 1. Sixteen male patients aged 27 to 72 years were given 0.5 to 1.5 avocados /day. Animal fat equivalent to a portion of added avocado fat was removed from the basal diet. 2. Serum total, free and ester cholesterol and phospholipids were measured twice a week during control and avocado feeding periods. 3. Of 16 patients during avocado feeding period, 8 gave significant decreases in total serum cholesterol from 8.7 to 42.8% and in phospholipids of from 12.5 to 21.0%. Decreases in ester form exceeded those in free cholesterol. 4. In no individual did cholesterol values rise during avocado feeding. Of 8 patients whose cholesterol values were unchanged, 3 were diabetic and one was hypercholesterolemic.

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Nidation in Progesterone-Treated, Estrogen-Deficient Hamsters, *Mesocricetus auratus* (Waterhouse).* (25723)

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Gestation in the mammal is divisible into 2 portions, the period of free and attached ovum. In a few animals such as bear, roe deer, Mustilids, seals, armadillo the period of free ovum may be extremely long, extending into months. In most mammals the free period is short and very definitely defined, but in mouse and rat, gestation, concurrent with lactation, may be prolonged, due to retardation in implantation associated with a large number of suckling young. The exact mechanism controlling extent of delay and induction of nidation in both types of delay cited above is still not clear. For a brief review of the literature the reader is referred to(1). Recently experimental studies have been made of factors inducing and controlling delay in non-lactating rats. The publications indicate that time of ovariectomy after fertilization, and kind, quantity, and time of administration of exogenous hormone are important factors. The adrenal has also been implicated. These variables may account for what appear to be inconsistent, contradictory results(2-10). Time of ovariectomy during period of natural delay of implantation in the armadillo also affects implantation and subsequent viability of embryos(11). Loeb(12) demonstrated that time of removal of corpora lutea before implantation in the guinea pig determines whether nidation will occur. Bloch(13) reported that progesterone induces implantation at normal time in non-lactating mice castrated after mating. In prepubertal mice, induced to ovulate and mate by administration of exogenous gonadotropins, implantation does not occur, blastocysts delay and have been recovered as late as 22 days; implantation can be induced by exogenous progesterone (Smithberg and Runner)(14). This paper describes results of experiments designed to determine effect of ovariectomy, adrenalectomy, hypophysectomy and progesterone and estrone on implantation in the golden hamster.

Methods and results. The golden hamster, unlike the rat, has a very regular estrous cycle (Ward(15)), and has no postpartum ovulation or estrus (Deanesly(16)). Moreover, the hamster has a very short progestational, or free-living stage in its gestation; implantation begins on day 5, at approximately 4 days and 8 hours developmental age (Ward (17)). All hamsters used were virgins of breeding age in which several cycles had been followed. Females in estrus were placed with males of proven fertility in the evening and left overnight. Copulations were observed in most and confirmed in all by presence of sperm the following morning, *i.e.*, day 1 of pregnancy. A group of 5 intact pregnant hamsters were given 2-4 mg of progesterone daily from days 2 or 3 of pregnancy to determine whether exogenous progesterone would prevent implantation. These animals were laparotomized on day 8 and the number of sites recorded. One animal was killed at this time, another killed at time of normal parturition (15 days and 12 hours—as determined from approximate time of ovulation, 2 a.m. of morning when sperm were found, (Ward(15,17)), and the remaining 3 were treated until day 20 and killed. Observations at laparotomy indicate no effect on time of implantation. These results agree with those obtained by Samelwitz, Dziuk and Nalbandov (20), and Cochrane and Meyer(9), who administered 4-16 mg of progesterone daily for variable periods after mating and obtained no significant effect on implantation and development. The remaining females were subjected to surgery at known period of pregnancy prior to implantation. Beginning at time of surgery they were maintained with, or without (control groups), daily injections of progesterone, and laparotomized when conceptual swellings would be easily visible if implantation had occurred. When implantations were found, the

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TABLE I. Effect of Various Treatments on Implantation in Hamster.

No. of animals	Day of surgery	Daily treatment before laparotomy	Day of laparotomy	No. of animals without sites at laparotomy	No. of animals with sites at laparotomy	Daily treatment after laparotomy	Day of autopsy	No. animals without sites at autopsy
Group A—Ovariectomized, progesterone given from day of operation to autopsy								
1	1	2 mg prog.	7	1	0	2 mg prog. + 1 γ estrone	11	1
3	1	4	7	1*	2‡	4 mg prog. + 1 γ estrone	11	1
1	2	2	7	0	1‡	0		0
11	2	4	7–9	1*	10§	4 mg prog. + 1 γ estrone	13–14	1
1	3	2	7	0	1‡	0	7	0
9	3	4	7–10	0	9‖	0	7 or 17	0
1	4	2	7	0	1‡	0	7	0
1	4	4	7	0	1‡	0	7	0
Group B—Ovariectomized and adrenalectomized								
5	2	4 mg prog.	6	1*	4‡	4 mg prog. + 1 γ estrone	11	1
Group C—Ovariectomized, no progesterone given								
4	2	0	9	4	0	1 γ estrone	11	4
1	3	0	7	1‡	0			1
2	4	0	6 & 8	2‡	0			2
Group D—Pituitary autograph to kidney								
4	2	0	10–11	4†	0	1 γ estrone	16–17	4
2	3	0	10–11	2†	0	*Idem*	17	2
1	2	0	Both horns flushed day 4, 56 hr after operation. No blastocysts recovered.					
Group E—Hypophysectomized only								
8	2	0	7–10	6	2‡	1 γ estrone	13–17	6
Group F—Hypophysectomized and given progesterone								
4	2	4 mg	9		4‡			

* 1 horn flushed at time of laparotomy.
† 1 animal of group had one horn flushed at time of laparotomy.
‡ Killed.
§ 9 pregnant animals carried to day 13-14, 1 non-pregnant animal carried to day 13, no implantation occurred.
‖ 4 carried to day 17, 5 killed at time of 1st laparotomy.

animal was usually killed. Number and conditions of implantations were recorded and the tracts fixed in AFA (3 parts 95% alcohol, 1 part commercial formalin, 1 part glacial acetic, and 5 parts water), dehydrated in alcohol and cleared through benzol to benzyl benzoate. After clearing they were re-examined for completeness of ovariectomy and correspondence of development to stage when killed. Variation in experimental design is shown in Table I, which also summarizes procedures and results. Animals in Table I are divided into the following groups: Group A: Bilateral ovariectomy plus 2 or 4 mg of progesterone daily. Group B: Bilateral ovariectomy and adrenalectomy plus 4 mg of progesterone daily. Group C: Bilateral ovariectomy; no progesterone. Group D: Hypophysectomy and pituitary autograft to kidney capsule; no progesterone. Group E: Hypophysectomy; no progesterone. Group F: Hypophysectomy plus 4 mg progesterone daily. Ovaries and adrenals were removed by usual methods. Unilateral hysterectomies and laparotomies were made by ventral approach. Hypophysectomy was done by parapharyngeal approach under ether and semisterile conditions. When pituitary gland was to be transplanted, it was sucked into a sterile cannula and placed in 0.9% saline at room temperature. Autografts of pituitary were made by placing the gland under left kidney capsule following method of Everett(18). The sellaturcica of hypophysectomized animals was examined with magnifying lens at autopsy to determine whether removal was complete.

Daily weights were recorded for all animals. Synthetic U.S.P. progesterone was used throughout. Two or 4 mg of progesterone, or a combination of progesterone plus 1 γ of estrone, was injected daily subcutaneously in .25 cc of corn oil. Hamsters were maintained on Rockland Rat or Purina Laboratory Chow. Corn sugar was added to drinking water 3-4 days after hypophysectomy. Adrenalectomized hamsters were provided with 0.9% saline until autopsy. In 13 instances where implantation sites were present after bilateral ovariectomy, animals were maintained after laparotomy on the same hormonal treatment as that given before laparotomy; 9 to late gestation; 4 to prolonged gestation. At autopsy, number and condition of implantation sites and conceptuses were recorded. Where no implantation sites were found at laparotomy, the same prelaparotomy regime plus 1 γ of estrone daily was given for additional 4-6 days. One uterine horn from 5 of these animals was flushed at time of laparotomy, *i.e.*, 2 of the 3 from Group A, 1 from Group B, and 1 from each of the first 2 subgroups of Group D. Both horns from one animal in Group D were flushed on day 4, approximately one day prior to time of expected implantation (Ward(17)), to determine how long the blastocysts would remain viable. A similar attempt was made to determine condition of the blastocysts in the control Group C, where one animal castrated on day 4 was laparotomized and the uterus flushed on day 6, approximately one day after implantation. Since small amounts of estrone added to progesterone cause implantation in the non-lactating rat, the finding that 89% of hamsters ovariectomized and given progesterone alone implanted led us to eliminate all possible exogenous estrogen (from food and progesterone solvent), Orsini and Meyer(19). For this 18 hamsters were placed on a diet of water and glucose alone from day 2 to day 7. Sixteen were ovariectomized and given 4 mg of progesterone daily, 6 received it in corn oil, 5 suspended in saline, 5 dissolved in Trioctanoin, a synthetic oil obtained from Eastman Kodak. The remaining 2 were intact, serving as controls on the effect of the diet alone. All showed implantation sites comparable to the normal stage of development when laparotomized on day 7.

Discussion. In Group A of Table I, 3 or 11% of animals ovariectomized and given progesterone failed to implant. It is possible that the oviduct may have been traumatized during the operation. Eleven of total ovariectomized females had unilateral pregnancies suspected to be attributable to this cause, as the number of conceptuses in the single horn was not sufficient to suggest unilateral ovulations.

In Group E 6 of 8 animals hypophysectomized failed to implant. No pituitary fragments were found in 8 animals. It is possible, however, that fragments too small to be detected accounted for implantation in the remaining two.

Progesterone in doses used caused no detectable delay in nidation in hamsters made estrogen deficient by hypophysectomy, ovariectomy, or ovariectomy and adrenalectomy, (Table I, Groups A, B, and F). These findings support the concept that estrogen is not required for implantation in the hamster, whereas in the rat progesterone alone prevents nidation and estrogen induces it(4,5,9,10). In the hamster estrogen produced at ovulation and immediately thereafter may be sufficient for progesterone alone to produce implantation subsequently. The work of Loeb(12), who found that removal of corpora from the guinea pig during first 2 days was not followed by implantation, but that removal of corpora at 75 hours was followed by implantation and apparently normal development, and Bloch (13), who was able to secure implantation in castrate non-lactating mouse by progesterone alone, supports this possibility. Both suggest that the ovary may sensitize the uterus in this early stage. In prepubertal mice in which mating and ovulation were induced by gonadotrophins evidence was found for both estrogen and progesterone secretion during the first 2 days of gestation, Smithberg and Runner (14). Subsequently in their untreated animals, the corpora degenerate, the blastocysts survive but do not implant. However, if these animals are given progesterone daily, implantation occurs.

Group D, with hypophysectomy and autograft to the kidney in the hamster is unlike that in the rat(18,21,10). Whether failure to

implant in the hamster under these conditions is due to a lack of luteotropin or luteinizing hormone has not been determined.

Cleavage stages, morulae and early blastocysts were flushed from the uteri of normal hamsters prior to implantation, yet in no case were ova or blastocysts recovered from the few experimental animals flushed before or after expected time of normal implantation. No evidence was found of abortive implantations in cleared, non-pregnant tracts. Moreover, implantations found in pregnant tracts (progesterone treated) appeared comparable to stages in the normal hamster. This suggests that without progesterone ova or blastocysts degenerate; at what stage is not known.

In experiment designed to eliminate possible estrogen contamination the entire group of animals lost weight when maintained on glucose, but all showed implantations demonstrating that exogenous estrogen is not necessary for implantation in the hamster. One possible source for contamination is in the progesterone itself. This, however, does not seem probable.

In no instance where implantation had not occurred at time of laparotomy, regardless of treatment, was it subsequently induced by estrone. This suggests that blastocysts were not alive at time estrone was begun.

Summary. 1. In the hamster, when ovarian hormones are lacking, due to ovariectomy, or hypophysectomy, implantation does not occur and blastocysts are not recovered. 2. Implantation does not occur in hypophysectomized hamsters with pituitary autografts. 3. Doses of progesterone, 2-4 mg daily, do not affect time of implantation in the intact hamster. 4. Implantation occurs with no apparent delay in hamsters made estrogen-deficient by ovariectomy, ovariectomy and adrenalectomy, or hypophysectomy and given daily injections of 2-4 mg progesterone. In experiments designed to eliminate estrogen in injection media and diet, implantation also occurred when progesterone was given to ovariectomized hamsters. This suggests that progesterone alone is required for maintenance of blastocyst and nidation in the hamster.

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Stimulatory Action of Adrenal Medulla and Catecholamines upon Hydroxylation of Steroids by Adrenocortical Homogenates. (25724)

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During comparative study of steroid synthesis by slices of human, canine and bovine adrenal glands incubated in autologous plasma, it was observed that slices containing cortical and medullary tissue produced more 17α,21-dihydroxycorticosteroids than slices containing cortical tissue alone. Medullary slices by themselves, when incubated under identical conditions, did not produce detectable amounts of dihydroxycorticosteroids. However, addition of such slices or of homogenized medullary tissue to incubates of adrenal cortex slices resulted in markedly increased synthesis of corticosteroids. To study the mechanism of accelerating effect of adrenal medulla, a simpler and better reproducible assay system than the slice-plasma system was needed. This report deals with effect of medullary homogenates on conversion of progesterone to 17α, 21-dihydroxycorticosteroids by bovine adrenal cortical homogenates in a Ringer-bicarbonate bovine albumin medium. Comparative studies of the effect of catecholamines on corticosteroid production by cortical homogenates are also reported.

Method. Steer adrenals, removed from carcass within 15 to 20 minutes after animal's death, were packed in cracked ice and brought to cold room of laboratory. The capsules were dissected, glands sliced and slices carefully freed of medullary tissue. Cortical and medullary tissue samples were then separately homogenized in double-distilled water in glass homogenizers equipped with Teflon pestles. 0.5 ml of cortical homogenate (equivalent to 100 to 125 mg of cortical tissue) was added to 125 ml Erlenmeyer flasks containing following ingredients:† (1) 0.5 mg (1.64 μmole) progesterone dissolved in alcohol and evaporated to dryness; (2) 7 ml of Krebs Ringer-bicarbonate medium containing 2% albumin; (3) 8 mg (24.2 μmole) of glucose-6-phosphate (disodium salt) and varying amounts of triphosphopyridine nucleotide (TPN). To selected flasks medullary homogenate or D (–) norepinephrine or D (–) epinephrine was added. Quantities studied are listed in Table I and Figures. Reaction volumes were equalized by addition of H_2O and usually were 8.5 ml. Flasks were gassed with 5% CO_2 in O_2 and shaken 4 hours at 37°C in Brunswick shaker. Contents of each flask were then extracted with 25 ml of chloroform and 17α,21-dihydroxycorticosteroids were estimated by method of Silber and Porter(1). Results of duplicate experiments agreed within $\pm$5%. In some experiments chloroform extracts were subjected to paper chromatography in system of Burton, Zaffaroni and Keutman(2). Steroids separating in cortisol region were quantitated by reaction with blue tetrazolium(3). These results were in good agreement with those obtained with procedure of Silber and Porter.

Results. Rates of formation of 17α,21-dihydroxycorticosteroids by homogenates of adrenal cortex and increments of rate due to addition of medullary homogenates are recorded in Table I. It is important to point out that there was no detectable steroid formation in absence of added progesterone. Likewise addition of glucose-6-phosphate and TPN was indispensable. The system represents thus hydroxylation of progesterone with the aid of TPNH regenerated by glucose-6-phosphate dehydrogenase of the homogenates.

Preliminary experiments had shown that concentrations of progesterone and glucose-6-

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† Organic compounds were obtained as follows: crystalline bovine albumin from Nutritional Biochemical; 1-norepinephrine bitartrate (levophed), 1-epinephrine bitartrate and 1-isoproterenol HCl from Winthrop-Stearns; L-Dopa and 3-OH tyramine HCl from Mann Research Labs, 3-OH tyramine from California Corp. for Biochem. Research. All others from Sigma Chem. Corp.

TABLE I. Effect of Medullary Homogenate on Production of 17α, 21-Dihydroxycorticosteroids by Adrenocortical Homogenates, in 13 Experiments.

	Cortex alone†	Cortex + medulla	
TPN,* mg	Rate,‡ μg/g/4 hr	Medulla added, mg	△ Rate,‡ μg/g/4 hr
16	99	34	+182
8	111	34	+153
4	111	34	+153
8	157	34	+121
8	222	38	+234
8	73	41	+185
8	370	41	+130
8	830	50	+172
4	349 ± 25	30	+116 ± 5
4	171 ± 1	40	+ 94
4	83 ± 2	60	+223 ± 10
4	191 ± 7.5	68	+304 ± 5
2	28 ± 2.3	31	+ 75 ± 0
2	19 ± 1.0	40	+ 61 ± 3
2	601 ± 13	50	+654 ± 50

* Sodium salt MW 837.

† Cortex homogenate equivalent to 115-125 mg of tissue.

‡ Basal rates, increments of rate (△ rate) due to addition of medulla, and deviations of duplicates from their mean are recorded.

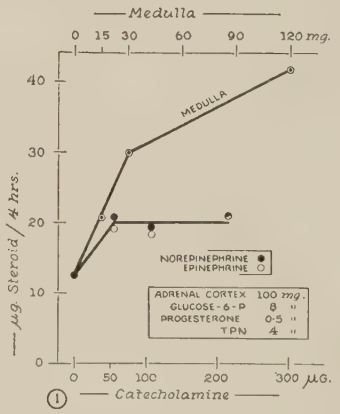
phosphate used provided maximal rate of steroid formation. Data in Table I show that TPN at concentration levels employed was not rate-limiting since there was no correlation between rate of steroid formation and amount of TPN added to the system. The great differences in rate of steroid formation of individual cortical homogenates were thus probably due to variation in enzymatic activity of adrenal glands obtained from abbatoir. Regardless of activity of the cortical homogenate, however, addition of medullary homogenate markedly increased rate of corticoid formation.

To explore the mechanism of accelerating action of medullary homogenates, the effect of catecholamines on corticoid production of cortical homogenates was studied. Fig. 1 shows results of representative experiment in which effects of various concentrations of D-epinephrine, D-norepinephrine and medullary homogenate were compared.

The lower curve in Fig. 1 shows that addition of either of 2 catecholamines greatly accelerated corticosteroid formation of adrenocortical homogenates. Amounts required for maximum stimulation varied somewhat among individual glands studied but never exceeded 100 μg of either D-norepinephrine or D-epinephrine.

Stimulation of steroid formation of adrenocortical homogenate by increasing amounts of medullary homogenate (upper curve of Fig. 1) far exceeded maximum obtainable with catecholamines. The shape of response curve indicated that the medullary preparation contained at least 2 active agents responsible for the steep initial slope and slower, almost linear, rise beyond inclination point of curve. This point lay between 15 and 30 mg of medullary tissue added.

To evaluate the contribution of medullary



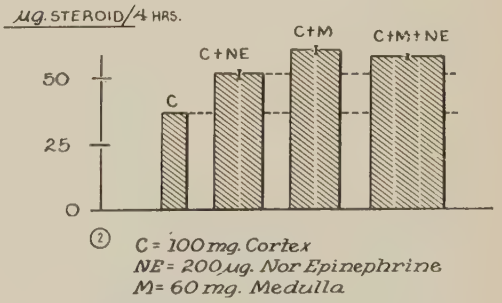
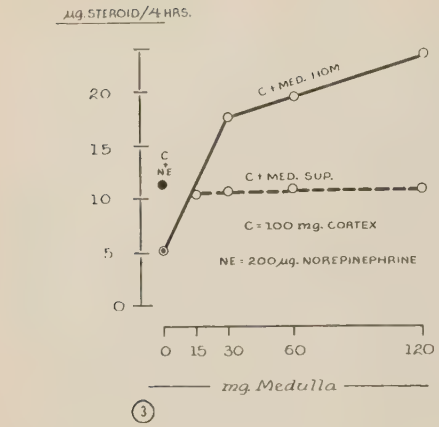
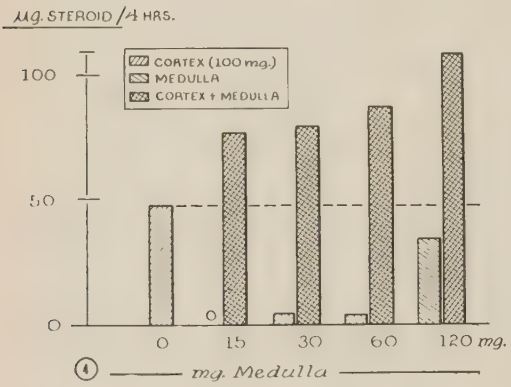


FIG. 1. Effect of increasing additions of medullary homogenates or catecholamines upon formation of dihydroxycorticosteroids by cortical homogenates. All flasks contained adrenocortical homogenate equivalent to 100 mg of cortical tissue and 4 mg (4.8 μmole) of TPN. Additions of medullary homogenate expressed as equivalent amounts of medullary tissue and of epinephrine or norepinephrine in terms of free amines (100 μg = 0.57 μmole) are indicated on top and bottom ordinates. All other additions as described under *Methods*. Total reaction volume = 8 ml.

FIG. 2. Effect of medullary homogenate and norepinephrine singly and combined on dihydroxycorticosteroid production. Designations as in legend to Fig. 1.





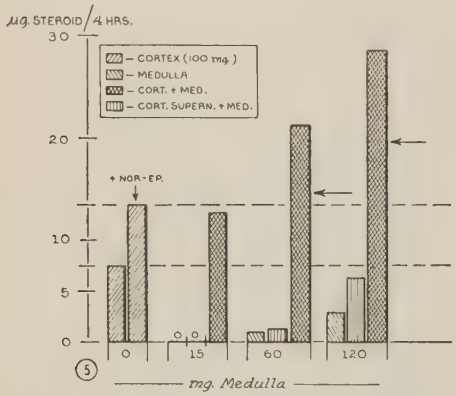


FIG. 3. Effects of increasing amounts of medullary homogenate and its supernatant fraction on steroid formation by cortical homogenates.

FIG. 4. Steroid formation by medullary homogenates and stimulatory action of medulla on cortical homogenates.

FIG. 5. Effect of supernatant fraction of cortical homogenate on steroid formation by medullary homogenate relationship to stimulatory activity of medulla.

catecholamines to the response curve, 2 types of experiments were performed (Fig. 2, 3). Fig. 2 shows that norepinephrine failed to accelerate steroid formation of cortical homogenate already stimulated by medullary homogenate; an indication that the system was saturated with respect to catecholamines. Since it could be expected that the catecholamines would be present largely in the soluble fraction of distilled water homogenates of adrenal medulla, the effect of supernatant liquid obtained by high-speed centrifugation (100,000 × g, 60 min) was compared with that of whole homogenate (Fig. 3). The response curve to increasing amounts of supernatant liquid was undistinguishable from that to increasing amounts of catecholamines (Fig. 1). Rate of steroid formation virtually coincided with that produced by saturating concentration of norepinephrine used as control (C + N E in Fig. 3). Catecholamine assays (method of Euler and Orwen(4)) upon some medullary homogenates used, gave values of 80-115 μg/15 mg of medulla, sufficient to produce maximum stimulation of corticosteroid synthesis obtainable with catecholamines. Hence the steep initial rise of response curves to medullary homogenates (Fig. 1 and 3), can be ascribed mainly to action of medullary catecholamines. The slower rise beyond the inclination point of curves was due in part to contamination of the medulla with cortical tissue derived from folds of the cortex which penetrate into the medulla.

The contribution of cortical remnants to stimulating action of medullary homogenates on steroid synthesis of cortical preparations was not readily noticeable since biosynthetic activity of homogenates ceased to be proportional to quantity of tissue used when concentration of tissue became low. Fig. 4 shows that at concentrations up to 60 mg of tissue/8.5 ml of medium, medullary homogenates by themselves produced negligible amounts of corticosteroids whereas twice this concentration synthesized about 70% as much steroid as 100 mg of cortical tissue in absence of added catecholamines. A similar, though less striking, dilution effect was found with cortical homogenates.

In experiment Fig. 5 special care was taken

to remove particles of cortex-like color from dissected and minced adrenal medulla. Steroid formation of this medullary homogenate was lower than that of previous preparations of unselected medullary tissue. Yet its stimulatory action on steroid formation of cortical homogenate was far greater than could be accounted for by activity of cortical remnants introduced with the medulla and stimulation of cortical homogenate by medullary catecholamines.

Since it appeared possible that activity of cortical remnants in medullary homogenates was limited by lack of soluble enzymes and cofactors present in the more concentrated cortical homogenates, one concentration series of medullary homogenates was supplemented with the soluble fraction of 100 mg of cortical homogenate obtained by high-speed centrifugation. Fig. 5 shows that this procedure failed to abolish the dilution effect although it did augment steroid formation. Arrows in Fig. 5 serve to indicate that 70% of total steroid formation can be accounted for by additive action of medullary cortical remnants and catecholamines. It thus appears that the stimulatory effect of medullary homogenates of corticosteroid formation of cortical homogenates is due to 3 factors: 1) medullary catecholamines, 2) cortical remnants, and 3) an interaction of unknown nature between particulate components of medullary and cortical homogenates.

Discussion. Hayano and Dorfman(5,6) reported that 11β-hydroxylating activity of bovine adrenal medulla was almost as high as that of cortex. Kahnt(7), on the other hand, failed to detect medullary activity. Microscopic examination of serial sections of bovine adrenal glands reveals that medulla invariably contains islands or strands of cortical tissue. Our experiments showed that hydroxylating activity of such cortical remnants becomes manifest only when comparatively high concentrations of medullary homogenates are assayed since activity/unit weight of homogenized cortical tissue decreases with increasing dilution of the assay system. Conflicting results regarding hydroxylating activity of medullary preparations can thus be explained as due to varying degrees of contamination with cortical tissue and operation of the dilution effect.

Our most interesting finding is the pronounced acceleration of conversion of progesterone to 17α,21-dihydroxycorticosteroids when small amounts of medullary homogenates were added to adrenocortical homogenates. Under these conditions hydroxylating activity of cortical remnants in medullary homogenates can be disregarded since the supernatant fraction which is free of such activity produced as high stimulations as whole homogenate. Significantly, epinephrine or norepinephrine effected accelerations of rate of hydroxylation of a similar order of magnitude as small amounts of whole medullary homogenates or their supernatant fractions. The quantity of catecholamines required matched satisfactorily total catecholamine content of medullary samples of equal stimulatory activity. Unpublished data indicate that isopropylnorepinephrine (isoproterenol), the third medullary catecholamine, and catecholamine precursor, dihydroxyphenylalanine were equally effective. In contrast, dihydroxyphenylethylamine (3-hydroxytyramine) considered the immediate precursor of norepinephrine(8), did not stimulate. No explanation of this discrepancy can now be offered. Dihydroxyphenylserine, an alternate potential precursor of norepinephrine, has not been tested. Adrenochrome is ineffective at low concentrations and inhibitory at higher concentrations.

While in majority of experiments only conversion of progesterone to 17α,21-dihydroxycorticosteroids was determined by the Silber-Porter reaction, paper-chromatographic separations of conversion products have been performed in exploratory experiments. Progesterone as well as derivatives hydroxylated at the C-17 and/or C-21 position were employed as substrates. With progesterone main reaction products were cortisol and corticosterone, the amount of the latter being about 3 times that of the former. 17α-progesterone yielded mainly cortisol. With either substrate, quantity of all reaction products was markedly increased by medullary homogenates, epinephrine or norepinephrine. There was no detectable change in relative

amounts of reaction products. Conversion of 11-deoxycorticosterone was not significantly accelerated by norepinephrine. It thus appears that catecholamines accelerate hydroxylations at C-21 and, perhaps, C-17 but not at C-11. This may explain Kahnt's(7) failure to detect an effect of medullary homogenates or epinephrine on 11β-hydroxylation of substance S by bovine adrenocortical homogenates. Firmer information on site and mechanism of action of catecholamines will require separation of the 3 hydroxylase systems.

Summary. 1. Conversion of progesterone to 17α,21-dihydroxycorticosteroids by homogenates of bovine adrenal cortex is markedly stimulated by addition of small amounts (15 mg/100 mg cortex) of medullary homogenate. Larger amounts produce additional, though relatively smaller increments. 2. The soluble fraction of medullary homogenates equivalent to 15 mg of medulla stimulates as much as the corresponding amount of whole homogenate. Larger amounts of this supernatant fraction do not produce additional increments. 3. Increments similar to those obtainable with 15 mg of medullary homogenate or medullary supernates are produced with 100 μg or less of D(−)epinephrine, D(−)norepinephrine, or D(−)isopropyl-norepinephrine. No further increment results from larger additions. 4. Total catecholamine content of 15 mg of medullary homogenate or corresponding supernatant fraction ranged from 80-115 μg, indicating that the stimulatory action of small amounts of medulla can be ascribed mainly to catecholamines. Additional increments produced by larger amounts of medulla are apparently due to contamination with cortical remnants and, possibly, an additional factor of unknown nature. 5. Exploratory experiments suggest that catecholamines accelerate C-21 and, perhaps, C-17, but not C-11 hydroxylations.

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Parainfluenza 3 — Assay and Growth in Tissue Culture.* (25725)

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Parainfluenza 3 (HA1) causes human(1) and possibly bovine disease(2,3), and is a virus which may produce persistent infection in tissue cultures(4). The process of formation of multinucleated giant cells(5), and a plaque assay technic dependent on these cytopathic changes have been reported(6). We describe a plaque assay system based on hemadsorption characteristics of HA1. Data are presented on thermal stability, rate of adsorption to cells, growth characteristics, and variation in cytopathic response of different cell systems to HA1.

* Aided by grant from National Fn.

Materials and methods. Virus. Mill's strain of HA1 was obtained from Dr. Wallace Rowe. Virus pools of passages 11 through 13 in our laboratory were used. Hemadsorption tests were performed by exposing washed tissue cultures to 0.4% suspension of thrice washed guinea pig red cells in phosphate buffered saline, pH 7.3. In hemagglutination

TABLE I. Variation in Response of Different Cell Systems to HA1.

Cell strain	Species origin	Growth medium	Characteristics cytopathic changes	Hemadsorption
Lohi	Human	BME 90, Calf 10	—	+
"	"	Homologous serum 20, Yeast .1	+	+
CRP	Rabbit	*Idem*	—	+
CRE	"	"	—	+
ERK-1	?	"	+	+
ERK-2	"	"	—	+
Amnion	Human	"	—	+
HeLa	"	"	+	+
EE	"	"	+	+

and hemagglutination inhibition tests, 0.25 ml of virus plus 0.25 ml of saline or serum were mixed and incubated at room temperature 1 hour. Then 0.25 ml of 0.4% suspension of washed guinea pig red cells was added, the tubes placed at 4°C overnight and read by pattern. Rabbits were immunized for 7 weeks with 4 weekly I.V. injections of virus and 2 I.M. injections of virus with adjuvant (Arlacel A, 1 part, Bayol F, 4 parts). Lohi cells (5) were grown either in Eagle's basal medium (BME) 90%, calf serum 10%, or Hanks BSS 80%, yeast extract 0.1%, human serum 20%(7). Growth conditions for cells tested for susceptibility have been published (8). After virus inoculation all cells were maintained in BME 75%, tryptose phosphate 20%, and rabbit serum 5%. All media contained penicillin 100 units and streptomycin 100 μg/ml. *Plaque assay.* Two-ounce tablet plaque bottles containing cell monolayers were washed 3 times with BSS and shaken free of residual fluid. The bottles were inoculated with 0.1 ml of suitable dilution of virus and rocked intermittently 1 hour at room temperature. Monolayers were overlaid with maintenance medium containing 0.6% nutrient agar and incubated 3 days; then the agar was poured off, 5 ml of 0.4% suspension of guinea pig red cells applied to each plaque bottle and settled at 4°C for 30 minutes. Red cells were rinsed off gently leaving small 1 to 2 mm "hemadsorption plaques." Plaque reduction by antibody was measured by adding 0.1 ml of serum to 1 ml of virus containing approximately 5 x 10^4 pfu. After incubation at room temperature for 1 hour, plaque bottles were inoculated with 0.1 ml of 1:10 dilution of this mixture. As pointed out by Holland(9) an important point in procedures in which the agar is poured off is the inclusion of calf serum in growth medium to prevent easy detachment of cells.

Results. Tissue cultures infected with HA1 may produce multinucleated giant cells, the centers of which become detached from the glass. This sequence of changes is the basis of plaque technic described by Deibel(6). Lohi cells grown in BME with 10% calf serum showed less marked cytopathic change than Lohi cells grown in BSS 80%, yeast extract 0.1%, and human serum 20%. Cells grown in calf serum medium were not satisfactory for plaque assay systems dependent on development of characteristic cytopathic changes, but were as sensitive as cells grown in the human serum medium for "hemadsorption plaque" procedure. Increasing dilutions of virus resulted in a linear decrease in number of plaques.

Variation in cell response. Four tube cultures of each cell strain shown in Table I were washed and inoculated with approximately 100 pfu of HA1 in 1 ml of maintenance media. After 3 days incubation 2 tubes of each set were stained with 1% crystal violet and examined for characteristic multinuclear giant cells. The remaining 2 tubes were used for hemadsorption tests. Results (Table I) show that although virus was detected in each instance by the hemadsorption test only 4 lines showed marked cytopathic changes. In many instances infected cultures were not easily distinguishable from control cultures.

Thermal stability. HA1 in maintenance medium was rapidly inactivated at elevated

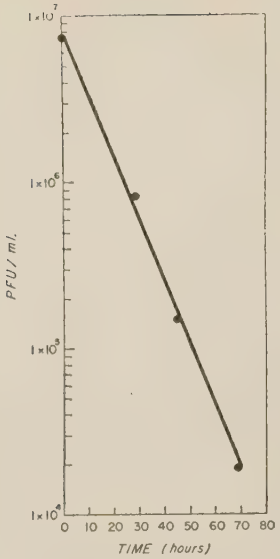

FIG. 1. Thermal inactivation 37°C.

temperatures. Less than 1% of infectivity survived 56°C for 15 minutes and approximately 50% remained after exposure to 45° for one hour. Fig. 1 shows decrease in titers during 39 hours incubation at 37°C.

Adsorption rate. Plaque bottles were shaken free of residual fluid and inoculated with 0.1 ml of suitable dilution of HA1 to yield 30-100 plaques/bottle for each adsorption period. At the end of each period, unadsorbed virus was removed by washing each bottle twice with 10 ml of cold BSS. Monolayers were overlaid with 5 ml of 0.6% agar in maintenance medium, incubated 3 days and number of plaques counted. Percent unadsorbed virus (Fig. 2) was calculated by subtracting plaque count for each adsorption period from count after adsorption for one hour and dividing by 100. Since plaque counts after 2 hours adsorption were less than 10% greater than counts after one hour, the latter were used routinely.

Evidence of antibody in sera used in tissue culture. Reports of antibody to HA1 in humans(1), cattle(2,3), and guinea pigs(10) necessitate pretesting of all sera with this virus. Data in Tables II and III show that sera from each of 8 calves, from pooled calf and from pooled human sera showed evidence of viral inhibition. All sera were trypsin treated and heat inactivated. The possibility that viral neutralization by serum in these tests is due to antibody is suggested by the wide range of titers, lack of decrease in titers after trypsin treatment, heat inactivation, or acetone extraction, and presence of inhibitor in the 20% sodium sulphate precipitated "gammaglobulin," prepared as described by Thurston *et al.*(11). We have not encountered inhibitors in our rabbit sera. However, each of 3 guinea pigs given a single intranasal injection of 2 x 10^6 pfu of HA1 developed significant levels of antibody, suggesting the possibility of natural undetected infection of laboratory animals with this virus. Table III shows hemagglutination inhibition titers, neutralization titers, and plaque reduction results

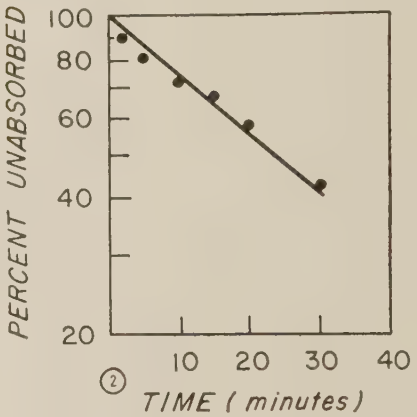

FIG. 2. Adsorption of HA1 to Lohi cells.

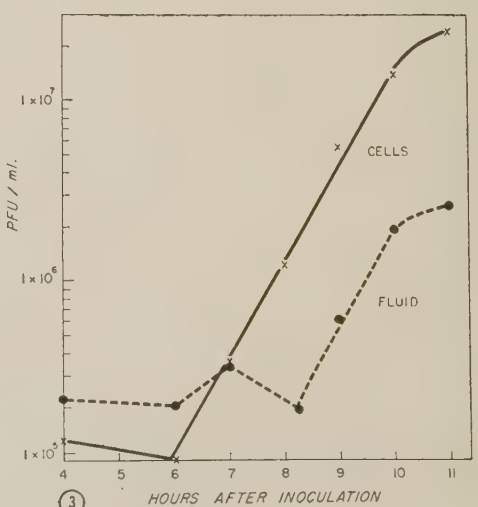

FIG. 3. HA1 growth curve. ––●–– in supernatant fluid. —×— in cells disrupted in 5 ml fluid.

with individual sera from 8 calves all under 6 months of age. Tube dilution neutralization results were obtained by mixing 0.5 ml of 4 fold dilutions of serum with 0.5 ml of virus containing 10^3 TCD_{50}/0.1 ml. After one hour incubation at room temperature, 0.2 ml of the mixture was added to cultures of Lohi cells. After 3 days incubation infected controls showed confluent hemadsorption when guinea pig red cells were added. The result with serum virus mixtures varied from no evidence of hemadsorption (complete neutralization) to complete coverage of monolayers by red cells. Those serum dilutions limiting hemadsorption to less than 50% of area of the monolayer are arbitrarily shown as exhibiting partial neutralization. Although the 3 methods are in general agreement in measuring viral inhibition, the sensitivity of plaque reduction method is demonstrated in results with serum from calf number 4.

Growth curve. Plaque bottles containing 2 x 10^6 Lohi cells grown in BSS 80%, yeast extract 0.1% and human serum 20% were washed 3 times and inoculated with 0.1 ml of virus containing approximately 6 x 10^6 pfu. After adsorption period of 1 hour, 5 ml of maintenance medium were added to each bottle and cultures incubated at 37°C for various intervals. Fluid was then removed from the bottle (fluid sample) and replaced by 5 ml of cold maintenance medium (cell sample) and both samples frozen and later assayed simultaneously by hemadsorption plaque technic. Fig. 3 shows results of this experiment. Starting 6 hours after inoculation or, after 5 hours incubation, titer of virus in cells increased logarithmically. Titer in the fluid, including any detached cells or cell debris, began to increase 2 hours after that in the cells. By 11 hours total virus in the fluid was only 1/10 that in cells. Thus concentration of virus per unit volume of packed cells was of the order of a thousand times that in the fluid. In this experiment an average of 50 pfu of virus was produced/cell.

TABLE II. Inhibition by Pooled Sera.

Serum	Hemagglutination inhibition	Plaque reduction No.	Plaque reduction % reduction
Calf	1:160	0	>99
" "gammaglobulin"	"	0	"
" "acetone extracted"	"	0	"
Human	1:40	0	"
Horse	1:10	>300	None det.*
Rabbit	<1:10	"	*Idem*
Expected (from control titration)		450	

* None detected.

TABLE III. Inhibition by Individual Calf Sera.

Neutralization titer Complete	Neutralization titer Partial	Hemagglutination inhibition	Plaque reduction No.	Plaque reduction % reduction
1:8	1:128	1:80	0	>99
1:32	"	1:40	1	"
1:8	1:32	1:20	0	"
<1:8	"	<1:10	19	95
"	"	1:40	8	98
1:512	>1:2048	>1:320	0	>99
1:8	1:128	1:40	2	"
1:32	"	1:160	4	99
Expected (from control titration)			480	

Rate of virus synthesis is a sensitive indicator of cell-virus relationship. Preliminary experiments suggest that the pattern shown in Fig. 3 may not hold for all other cell systems.

Summary. Various parameters involved in assay and growth of HA1 virus in tissue culture have been examined. Variation in occurrence of cytopathic changes in different cell systems led to development of a new plaque assay technic utilizing hemadsorption properties of the virus. HA1 is adsorbed to cell monolayers rapidly from small volumes (0.1 ml) so that 1 hour adsorption is adequate for routine plaque work. Thermal inactivation curve showed a halflife at 37°C of 10 hr. Presence of antibody to HA1 in human and calf serum, and in serum from guinea pigs inoculated intranasally confirms work by others and emphasizes the need to pretest all sera used in work with this virus. Growth curves showed that at end of logarithmic increase, concentration of virus in the supernatant fluid was less than 0.1% that in cells. An average of approximately 50 pfu was produced/cell.

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Mineralocorticoid Effects of 9α-Fluorodeoxycorticosterone in Adrenalectomized Rats.* (25726)

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A large number of 9α-halogenated derivatives of hydrocortisone, corticosterone, 11β-hydroxyprogesterone and 11β,17α-dihydroxyprogesterone and of corresponding 11-oxo steroids have been prepared from 11α-hydroxy intermediates(1,2). In addition to glucocorticoid effects, many of these compounds have shown high levels of mineralocorticoid and survival activity(2-7). So far, however, no biological data seem to be available for 9α-halogenated steroids lacking oxygen functions at carbon 11. From this standpoint, our tests with 9α-fluorodeoxycorticosterone acetate for mineralocorticoid effects in rats are believed to be of interest. The steroid was synthesized directly from corticosterone acetate by Bergstrom and Dodson(8).

Materials and methods. Male Sprague-Dawley rats (150-200 g) fed Purina Chow for about a week were adrenalectomized and maintained with sucrose cubes and tap water overnight. Approximately 24 hours following operation, the animals were injected subcutaneously with deoxycorticosterone acetate (DCA) or with various doses of 9α-fluorodeoxycorticosterone acetate (9α-F-DCA) in Mazola oil. Brisk heating readily dissolved 9α-F-DCA in oil. All animals received in addition a subcutaneous injection of 2.5 ml isotonic saline. Four-hour samples of urine were collected in metabolism cages and analyzed for Na and K content. The DCA-like property of 9α-F-DCA was evaluated by its influence on Na excretion and the ratio of Na/K. After preliminary tests, 9α-F-DCA and DCA were tested simultaneously in a 4-point comparative assay with 14 rats/point, and relative potencies were calculated by the method of Irwin as discussed by Pugsley(9). All measurements of urinary electrolytes have been converted to logarithms. Repeated standardization tests have revealed that this adjustment gives statistically suitable responses, with relatively constant standard error values over a wide dosage range of DCA. Analyses for urinary Na and K were done on Beckman flame photometer.

Results. Data from several initial tests with 9α-F-DCA are summarized in Table I, together with those of 65 untreated animals for convenient reference. DCA as a reference standard at 10 μg/rat in each test produced Na retention and a reduction of ratio of Na/K. A small K excretion accompanied these urinary changes. In these data, 9α-F-DCA produced greater effects on electrolytes over the range of 1-500 μg/rat than did the standard dose of DCA. However, 9α-halo steroid was less effective at dose of 0.3 μg than 10 μg of DCA, to suggest a potency of at least 10 but less than about 30 times rela-

* The authors thank Janet L. Wallen for technical assistance and D. W. Calhoun and Dr. V. A. Drill for helpful criticism and review of manuscript.

TABLE 1. Effects of Graded Amounts of 9α-F-DCA on Urinary Excretion of Na and K in Adrenalectomized Rats.

Treatment		Mean log response		
Compound	μg/rat	Na	K	(Na × 10)/K
		.78	.45	1.34
DCA	10	.44	.51	.93
9α-F-DCA	500	.28	.69	.59
"	50	.06	.49	.56
DCA	10	.46	.59	.88
9α-F-DCA	4	.32	.46	.86
"	1	.33	.64	.69
DCA	10	.36	.56	.80
9α-F-DCA	.3	.62	.68	.94
"	.1	.77	.51	1.25

Na and K = log meq/l after dilution of sample/rat/4 hr to 25 ml with water; pooled s = ± 0.22, ± 0.21 and ± 0.20 for Na, K and (Na × 10)/K, respectively; least significant difference (P = 0.05) relative to untreated controls (65 animals) = −0.14 for Na, +0.13 for K and −0.12 for Na/K response; 8-10 animals/treatment.

tive to DCA. Results of simultaneous assay with 2 doses each of DCA and 9α-F-DCA are shown in Table II. Using Na excretion as index of DCA-like activity, a relative potency of 12 was calculated for 9α-F-DCA, with 95% limits of 6.1 to 23.7. No consistent pattern of effects on K excretion was seen with the compounds. Fig. 1 shows data on Na/K. A relative potency of 14.3 (95% limits = 9.1-22.5) was obtained using response index of Na/K for 9α-halo steroid. From these limits, it follows that potency of 9α-F-DCA is significantly greater than that of DCA, with $P < 0.05$. Parallel regression lines in the Figure were drawn in accordance with the pooled slope of DCA and 9α-F-DCA. A chi-square test for parallelism of dose-response curves indicated validity of the assay(9).

Discussion. Our study demonstrates that 9α-F-DCA is a potent mineralocorticoid. The steroid possesses 12 and 14.3 times activity of DCA by response indices of Na retention and reduction of Na/K, respectively. It appears to possess, therefore, an interesting level of activity as found in other mineralocorticoids such as aldosterone(10,11), 19-nor-deoxycorticosterone(12) and various synthetic 9α-halo corticosteroids(2,3,5,11,13,14). Its closest structural relative in the series of 9α-halo steroids can be considered to be 9α-fluorocorticosterone acetate, which possesses 26.2 times the potency of DCA (95% confidence interval = 10.9-66.0) according to Na retention tests of Fried and Borman in rats(2,5). Other data on life maintenance activity in adrenalectomized animals also suggest a strong DCA-like property for this steroid(4,6). These data show that Na retaining activity may be increased by 2.2-fold with introduction of oxygen function at carbon 11 of 9α-F-DCA (potency ratio = 26.2/12 for 9α-fluorocorticosterone acetate/9α-F-DCA). However, the potency of 9α-fluorocorticosterone acetate is not significantly greater, statistically, than that of 12 reported above for 9α-F-DCA.† It would thus appear from progression of mineralocorticoid activities, from DCA, 9α-F-DCA to 9α-fluorocorticosterone acetate, that 9α-halogenation is more influential in increasing activity than 11β-hydroxylation.

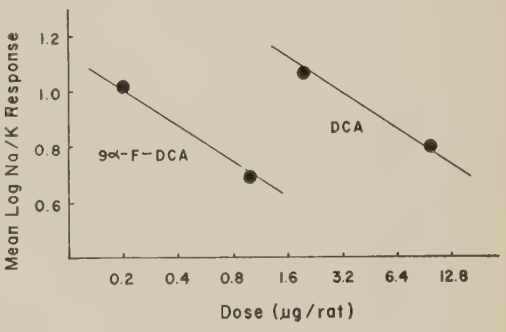

FIG. 1. Relative potencies of DCA and 9α-F-DCA as determined by ability to reduce Na/K response in adrenalectomized rats; potency of 9α-F-DCA = 14.3 (95% limits = 9.1-22.5) relative to DCA; composite slope for the 2 steroids = 0.42/10-fold increment of log dose; 14 rats/treatment.

TABLE II. Results of Assay for Mineralocorticoid Activity with 2 Dosage Levels of DCA and 9α-F-DCA in Adrenalectomized Rats.

Treatment		Mean log response	
Compound	μg/rat	Na	K
DCA	2	.71	.65
	10	.41	.63
9α-F-DCA	.2	.64	.63
	1.0	.41	.72

Potency = 12.0 (95% limits = 6.1-23.7) for Na response relative to DCA; Na and K = log meq/l after dilution of sample/rat/4 hr to 25 ml; 14 rats /treatment.

† A t value of 1.4 corresponding to P = 0.15 was computed with data for 9α-fluorocorticosterone acetate(2,5) and for 9α-F-DCA from our study.

Fried and Borman speculated on possible role of 9α-halo modifications in glucocorticoids (2). Data were presented to demonstrate parallelism between glucocorticoid activity and electronegativity, or ability of 9α-halo substituents to strengthen acidity of adjacent 11β-hydroxy radicals in steroids. However, that 9α-F-DCA is 12-14 times as potent as DCA would suggest other factors besides the influence of electronegativity must account for increased mineralocorticoid effects with 9α-halogenation.

Summary. The mineralocorticoid effects of 9α-fluorodeoxycorticosterone acetate were investigated in adrenalectomized rats. A quantitative 4-point assay gave potency estimates of 12 and 14.3 for the steroid relative to DCA, as judged by reduction of urinary Na excretion and the ratio of Na/K, respectively. Thus, addition of a 9α-fluoro radical can increase mineralocorticoid activity in a steroid lacking oxygen function at carbon 11. These and other published data suggest that 9α-halogenation plays a singularly important part in high DCA-like activity of various 9α-halo corticosteroids.

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Intestinal Absorption of a Modified Heparin.* (25727)

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Schanker *et al.*(1) and Hogben *et al.*(2) have shown that intestinal absorption of many drugs is in part dependent on the state of ionization of the drugs. Drugs which are weak organic acids or bases were more completely absorbed from the gastro-intestinal tract in pH ranges which favor the unionized radical. Loomis(3) has shown that sodium heparin is absorbed from the duodenal lumen of the dog at pH 4 but not at higher pH. Warner(4) believes that glucuronic acid carboxyl groups of chondroitin sulfate protonize between pH 5 and 2 thereby reducing ionization of the complex. A similar situation may exist for glucuronic acid carboxyl groups of heparin. If ionization is an important factor in regulating absorption from the intestine, then suitable modification of ionizing potentialities of heparin may enhance absorption in the physiological pH range. The current study is an attempt to accomplish this by esterification of heparin. A modified heparin has been prepared and evidence presented indicating that it is partially methylated and has undergone partial desulfation. It retains its original anticoagulant action and significant absorption

* This work supported by grant from Nat. Inst. of Health.

TABLE I. Elemental Analyses: Radioactive Samples Are Corrected for Background and Are Average of 2 Determinations.

	$Carbon^{14}$	Sulfur		Sodium	
Sodium heparin		11.4%	S.D. .34	9.9%	S.D. .24
Unpurified heparin derivative	125c/m/30 mg	10.4	" .94	7.9	" .24
Purified heparin derivative	116c/m/30 mg	10.6	" .41		

from the intestine occurs at pH 5 and 6.

Methods. Powdered sodium heparin† was suspended in methanolic-HCl (96.5% methanol, 3.5% 1N HCl). The suspension was kept at room temperature 1 hour, then centrifuged. The supernatant was discarded and the precipitate dried in vacuum at 0°C. The product was then powdered and used without further purification in animal experiments. Additional identical preparations were made using C^{14} methanol and the precipitate purified by washing with methanol and by reprecipitation from aqueous solution by addition of alcohol to 70% by volume. The precipitate was then dried. Analysis for C^{14}, S(5), and Na(6) were made on the unpurified product and analyses for C^{14} and S were made on the purified product. *In vitro* Lee-White (7) clotting times were performed on the modified product and compared to sodium heparin standards. Fourteen adult mongrel dogs were fasted 24 hours. Under pentobarbital (30 mg/kg) intravenous anesthesia, 15 to 20 cm length of duodenum located about 20 cm from the pylorus was exposed and transected at both ends leaving mesenteric blood supply intact. This loop of intestine was irrigated with approximately 200 ml of physiological saline and the distal end ligated. Undiluted MacIlwaine buffer(8) (Phosphate-citric acid) solutions containing the modified heparin were instilled into the loop and proximal end ligated. The segment was replaced in the abdomen and the incision closed with clamps. Systemic anticoagulant effect was determined by Lee-White(7) 3-tube method on femoral venous blood samples before and at ½ or 1 hour intervals following instillation of the solution in intestinal loop.

Results. Table I shows that purified C^{14} methanolic-HCl treated preparation of heparin retained much of its C^{14} activity indicating partial methylation of heparin. Sulfur content of both purified and unpurified product was decreased approximately 11.0% in comparison to sodium heparin. Sodium content of the unpurified product decreased approximately 20% compared to sodium heparin. Fig. 1 indicates *in vitro* anticoagulant effect of modified heparin was equal to that of original sodium heparin over a range of 0.5-5 μg. Table II indicates that 200 mg of modified heparin instilled into intestine with 20 ml of buffer having pH values of 5, 6, and 7 resulted in various degrees of anticoagulant effect for 5 hours. In presence of these buffers a significant anticoagulant effect was generally obtained within the first half hour. Both intensity and duration of anticoagulant effect diminish with rise in pH. The pH of luminal contents at end of experiments was 6 to 6.5 regardless of buffer used. Intestinal loops showed no gross evidence of general mucosal inflammation; however, there was a local inflammatory response at and adjacent to the area of site of application of ligature at each end of loop.

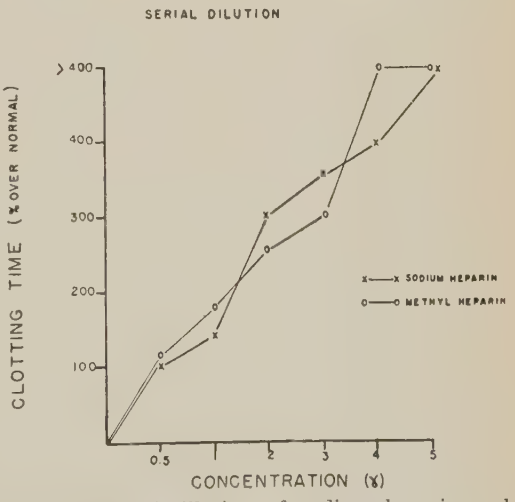

FIG. 1. Serial dilution of sodium heparin and modified heparin as measured by the Lee-White 3 tube method. Volumes in all tubes were 0.1 ml.

† Heparin was generously supplied by Eli Lilly and Co., Indianapolis.

TABLE II. Influence of Buffers at pH 5, 6, and 7 on Absorption of Modified Heparin Ester from Duodenal Loop of Dogs. Clotting time of venous blood recorded to nearest 10% of control. Each dog acted as its own control. Control clotting times were between 14 and 21 min. The number 0 indicates clotting time had fallen to, or below, control at that period. In dogs 1, 4, and 9 venous sampling was discontinued prior to cessation of anticoagulant effect. Figures of 400 represent values of 400% of control or above.

		Clotting time of blood as % of control										
		Hr after instillation										
Dog No.	Buffer pH	½	1	1½	2	2½	3	3½	4	4½	5	5½
1	5	180	400	400	400		400					
2	5	150	"	"		400		400	400	220	90	0
3	5	260	"	"	"	390		230	110	0		
4	5	190	390	"		400						
5	6	110	120		0							
6	6	400	400	"	400				0			
7	6	180	"	"	"		310		40			
8	6	130	"	300	130	30	0					
9	6	0		400	400		400	400		400		
10	7	160	"		"	400	"	280		0		
11	7	170	350	230	10	0						
12	7	0	220	210	130	30	0					
13	7	No effect										
14	7	60	0									

Discussion. The current lack of conclusive information regarding the structure of heparins precludes specifically identifying alterations produced in the modified heparin we used. Wolfrom and co-workers(9) represent a heparin as a tetrasaccharide unit composed of alternating glucosamine and glucuronic sugars containing 5 sulfonic acid residues. Two of these sulfate residues are linked to the NH_2 group of glucosamine. The other 3 are distributed as O-sulfate residues. Sodium heparin would be represented by one sodium atom/sulfate residue. Mild acid hydrolysis (10) involves cleavage of the labile sulfamic acid groups. This may account for the observed decrease in sulfur and sodium in the preparation we used. The O-sulfate groups undergo slower hydrolysis(10) and would not be expected to hydrolyse under the mild conditions we used. Each glucuronic acid unit has a terminal, free, carboxyl radical. Wilander(11) attributes acidity of heparin to the carboxyl group in accordance with data derived from titration curves. If carboxyl groups of heparin protonize between pH 5 and 2, as in chondroitin sulfate, and thereby render the molecule less ionized, this may explain observed absorption of sodium heparin at pH 4 described by Loomis(3). Partial methylation of these carboxyl radicals might account for the observed absorption of this compound at a more physiological pH. Minimal desulfation may also play a role in enhancing absorption at pH 5 and 6. However, as pH approaches neutrality the modified heparin is less significantly absorbed.

Using Loomis's(12) detoxication value for sodium heparin of 5 μg/kg/min, only approximately 5% of instilled modified heparin was absorbed in current experiments. If the pH of luminal contents was the sole factor regulating absorption, since some absorption occurred at pH of 6 to 7, the anticoagulant should continue to be absorbed at this pH range until absorption is more nearly complete. However, the pH of luminal contents at the end of experiments was 6.0 to 6.5 when systemic anticoagulant effect was not evident. Current experiments permitted absorption only from the segment of intestine containing heparin. Experiments of Schanker involved perfusion of entire small intestine of the rat until equilibrium was reached and then only approximately ½ of many drugs was involved. It is possible that presence of buffer salt is important in influencing absorption of the modified heparin by a mechanism other than its buffering action. Following progressive absorption of the buffer salt total ion concentration in the intestinal loop would be expected to change. Prior experiments have shown that no anticoagulant effect occurs following instillation of only the MacIlwaine buffer of pH 4 and 7.

Summary. A modified heparin has been prepared. This heparin derivative has undergone slight methylation and partial de-sulfation. Anticoagulant activity was retained, and limited absorption from duodenum of dogs as measured by systemic anticoagulant effect occurred following instillation of the modified heparin in buffer solutions of pH 5, 6, and 7.

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Further Observations on Mechanism of Hypoglycorrhachia in Experimental Meningitis.* (25728)

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We have demonstrated that incubation of CSF containing polymorphonuclear leukocytes with pneumococci at 37°C resulted in greater consumption of glucose(1) than when organisms were incubated in cell-free CSF. CSF containing a comparable number of leukocytes in absence of bacteria displayed little glycolytic activity. These findings pointed to definite synergistic effect between polymorphonuclear leukocytes and bacteria in depressing CSF glucose. The reaction was dependent upon number of bacteria and white cells in the incubation mixture but was not altered when erythrocytes were added to the medium. The mechanism of synergistic action of leukocytes and bacteria in enhancing glycolysis in the CSF was not clear, but it was suggested that bacterial cells multiplied more rapidly in the presence of leukocytes resulting in increased consumption of glucose, or that white cells engaged in phagocytosis exhibit greater glycolytic activity. Evidence will be presented here that the synergistic effect between cells and bacteria in producing hypoglycorrhachia is a function of enhanced phagocytosis in the subarachnoid space of intact animals.

Methods. Aseptic meningitis was produced in mongrel dogs by injection of 4 ml of sterile pyrogen-free, 0.85% NaCl into the cisterna magna. Four hours later 4 ml of CSF which regularly contained 2000 to 4000 WBC/mm^3 (95-99% polymorphonuclear leukocytes) was removed by cisternal puncture. The animals were then given 4 ml of physiological saline containing 10^8 viable pneumococci intrathecally and 3 hours later CSF was again removed for quantitative determinations of bacteria, leukocytes and glucose. One group of control animals was given physiological saline at beginning of experiment and 4 hours later,

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† Work performed during tenure of Lederle Medical Faculty Award.

and in another, pneumococcal meningitis was produced without antecedent aseptic meningitis. In other studies heat-killed pneumococci, India ink particles and a purified lipopolysaccharide obtained from *Salmonella abortus equi* (Pyrexal) were administered to animals with aseptic meningitis. Seed cultures of type III pneumococci were stored at –70°C. To prepare a culture for infection of animals, the contents of one tube were thawed and added to 9 ml tryptose phosphate broth. After incubation at 37°C for 8 hours, the broth culture was diluted with 0.85% NaCl solution. Inocula consisted of 4 ml suspension containing approximately 10^8 viable bacteria. Number of organisms in inocula and CSF was determined by colony counts of serial 10-fold dilutions, a single colony being counted as one viable unit. Leukocytes were counted in standard manner and CSF glucose was determined on cell-free supernates by the glucose-oxidase method(2).

Results. Eight dogs were given 0.85% NaCl intrathecally. Four hours later 2000 WBC/mm^3 had appeared in the CSF and no appreciable decrease in glucose had occurred (Fig. 1, top). A second cisternal puncture 3 hours later, revealed further increase in cells without significant drop in sugar. Administration of a second aliquot of 4 ml 0.85% NaCl 4 hours after beginning of experiment did not alter results. A second group, consisting of 9 animals, was given 10^8 pneumococci suspended in 4 ml 0.85% NaCl, after aseptic meningitis had developed (Fig. 1, center). Three hours later, CSF glucose of these animals had dropped from 82 to 28 mg %. Number of bacteria remained approximately the same as that in the original inoculum. While there had been an increase in number of leukocytes, the increment was no greater than in controls with aseptic meningitis in whom hypoglycorrhachia did not develop. In a third group of 8 dogs without aseptic meningitis, pneumococci were instilled into the cisterna magna and 3 hours later no significant fall in CSF glucose had occurred (Fig. 1, bottom). This contrasted sharply with findings in animals with aseptic meningitis antedating pneumococcal meningitis. Twenty-four hours later this difference was no longer apparent and the sugar was depressed in both groups of animals.

These observations indicate that a combination of pneumococci and leukocytes possesses greater glycolytic activity than either of its constituents *in vivo* as well as *in vitro*. Serum sugars were determined along with CSF sugars in all experiments and showed no appreciable deviation from normal.

To determine whether the decrease in CSF glucose could be attributed to alteration in metabolic requirements of the bacteria, the experiment was repeated employing pneumococci which had been rendered non-viable by exposure to 60°C for 30 minutes (Fig. 2). Six animals with antecedent aseptic meningitis, and approximately 2000 WBC/mm^3 in the CSF, experienced a marked fall in sugar when 4 ml suspension containing 10^8 non-viable pneumococci was injected (Fig. 2, bottom). On the other hand, hypoglycorrhachia did not ensue when the same number of bacteria were administered to animals without aseptic meningitis.

In another experiment 4 animals with aseptic meningitis were given 4 ml of 1% suspension of sterile, pyrogen-free India ink, and 4 ml of saline containing 1 μg of purified lipopolysaccharide Pyrexal was administered to 4 others. All 8 animals demonstrated a fall in glucose of from 50-90% of base-line values. In controls without aseptic meningitis injected with India ink and Pyrexal, there was a reduction in CSF glucose of only 10-20%.

Discussion. These data illustrate that glycolysis in CSF is brisker when bacteria are introduced into CSF containing leukocytes than when organisms are administered to animals with normal spinal fluid. Identical results were obtained when non-viable organisms were administered. This eliminates from consideration the possibility that viable bacteria consume glucose more avidly in presence of leukocytes, and suggests that some alteration in metabolism of the white cells is responsible for the hypoglycorrhachia. Introduction of both viable and non-viable pneumococci into CSF of animals with aseptic meningitis was associated with marked rise in *number* of leukocytes, but the relative increase was no greater than that observed dur-

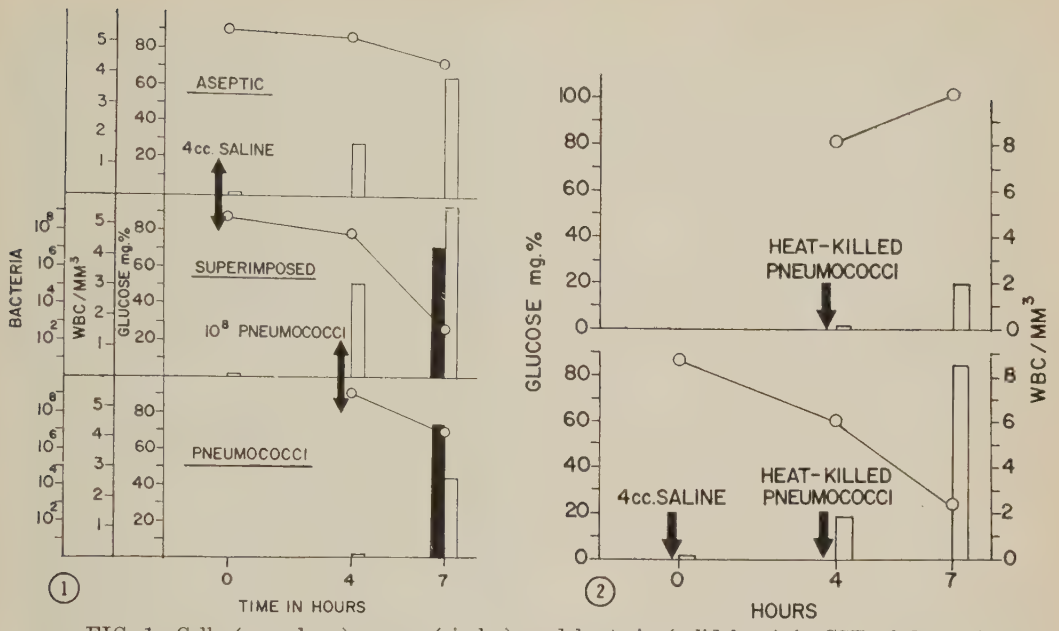

FIG. 1. Cells (open bars), sugar (circles), and bacteria (solid bars) in CSF of dogs with aseptic meningitis (top), aseptic meningitis with superimposed pneumococcal meningitis (center), and pneumococcal meningitis without antecedent aseptic meningitis (bottom).

FIG. 2. Effect of inj. of heat-killed pneumococci on CSF sugar (circles) and cells (bars) in dogs with aseptic meningitis (bottom) and normal controls (top).

ing aseptic meningitis produced by injection of saline alone. Furthermore, production of aseptic meningitis with streptokinasedornase (3) and penicillin or serum(4) has not resulted in hypoglycorrhachia despite appearance of pleocytosis far greater than that observed in our animals. It seems unlikely then that increase in number of leukocytes *per se* is responsible for reduction in glucose.

More attractive is the possibility that in presence of bacteria, leukocytes were stimulated to active phagocytosis. It has been shown that leukocytes engaged in phagocytosis of bacteria and inert particles manifest augmented consumption of glucose and oxygen and produce more lactic acid(5-7). Evidence that phagocytosis of bacteria by leukocytes played an important part in decrease in CSF glucose lies in the finding that administration of India ink particles to dogs with aseptic meningitis resulted in a comparable fall in CSF glucose. Most particles were visible within leukocytes and studies employing quantitative technics are now in progress to correlate degree of phagocytosis with hypoglycorrhachia.

The drop in glucose following intrathecal administration of a purified soluble bacterial endotoxin to dogs with aseptic meningitis is consistent with the hypothesis that hypoglycorrhachia is a consequence of phagocytosis. It has been demonstrated that granulocytes from animals and man given purified bacterial lipopolysaccharides possess an enhanced capacity for phagocytosis of staphylococci(8). In addition, increased glycolysis by granulocytes in contact with endotoxin has been described(9). Whether this is related to increase in cells' capacity for phagocytosis remains to be determined.

Summary. When dogs with aseptic meningitis were infected with pneumococci intrathecally, a profound drop in CSF glucose occurred which was not observed in control animals given pneumococci without antecedent production of aseptic meningitis, or in animals with aseptic meningitis in absence of superimposed bacterial infection. Hypoglycorrhachia also occurred when heat-killed pneumococci, India ink particles and bacterial endotoxin were administered to animals with aseptic meningitis. These data suggest that

the fall in CSF glucose is dependent upon presence of leukocytes engaged in active phagocytosis.

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Production of Cardiac Necroses and Nephrocalcinosis by Stress in Adrenalectomized Rats.* (25729)

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In various animal species, including the rat, large doses of certain corticoids and electrolytes produce massive "infarctoid" myocardial necroses, irrespective of presence of adrenals. Furthermore, small doses of corticoids and electrolytes, although by themselves ineffective, can so prepare or "condition" the myocardium that upon subsequent exposure to stressors (*e.g.,* cold, surgical trauma, restraint or forced muscular exercise) it responds with acute development of a usually fatal, infarctoid cardiopathy. In all these circumstances, the cardiac lesions are accompanied by nephrocalcinosis if Na_2HPO_4 is used as conditioning electrolyte(1). In previous studies, soon after it became clear that numerous manifestations of the alarm reaction result from discharge of ACTH and corticoids, it was shown that not all stress-induced changes are relayed through the hypophyseal-adrenal system, since many of them occur even after hypophysectomy(2) and adrenalectomy(3). It seemed of interest to determine, therefore, whether induction by stress of an infarctoid cardiopathy and of nephrocalcinosis in suitably conditioned rats is dependent upon integrity of the adrenals.

Materials and methods. Eighty female Holtzman rats, with mean initial body weight of 100 g (range: 94-110 g), were subdivided into 8 equal groups and treated as indicated in Table I. Adrenalectomy was performed on first day of experiment, under ether anesthesia. Na_2HPO_4 was given at dose of 1 mM in 2 ml of water twice daily by stomach tube. Triamcinolone§ or 9α-fluorocortisol (F-COL)‖ acetate was administered as microcrystal suspension, subcutaneously, at daily dose of 500 μg in 0.2 ml of water, during first 4 days; then, the daily dose of both these steroids was raised to 750 μg. Treatment with various stressors was initiated on sixth day. The animals were exposed to cold (3°C) 19 hours in a refrigerated room. Motor denervation of all 4 extremities was performed under ether anesthesia. Restraint procedure consisted in tying the rats to boards with adhesive tape, in prone position, for 7 hours. A detailed description of all these technics has been given elsewhere (1). Throughout period of observation the animals were fed exclusively on Purina Fox Chow. The experiment was terminated on eighth day by killing all surviving rats with chloroform. Immediately after autopsy, hearts and kidneys were fixed in alcohol-for-

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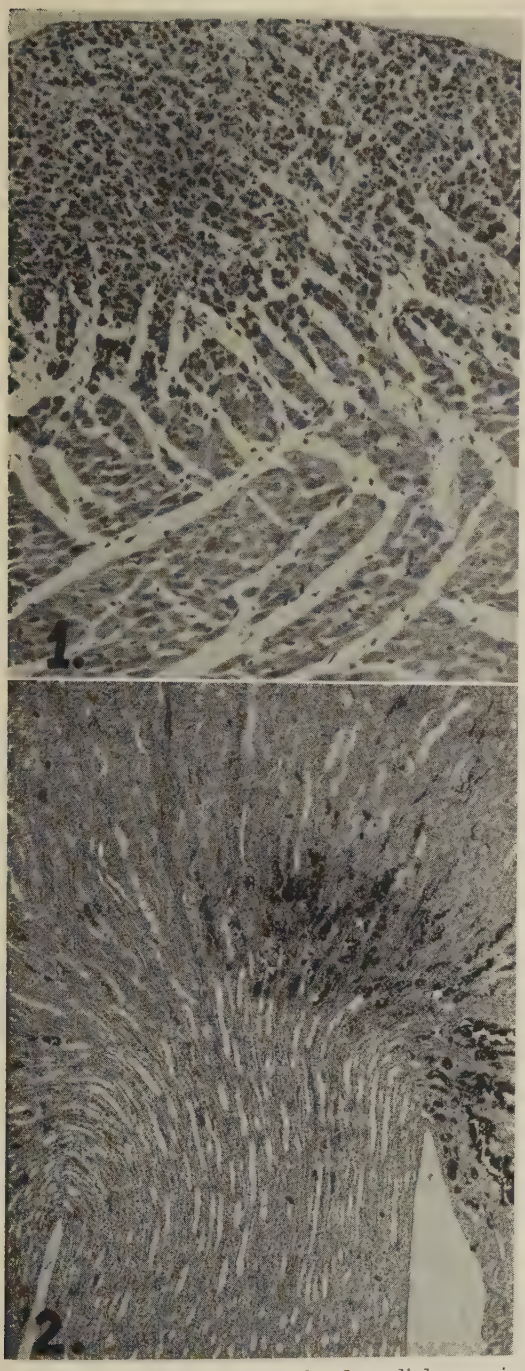

FIG. 1. Extensive, acute subendocardial necrosis with secondary inflammatory reaction in myocardium of rat of Group 2 (hematoxylin-phloxine × 100).

FIG. 2. Widespread nephrocalcinosis at corticomedullary junction line of kidney in rat of Group 3 (von Kóssa technic with hematoxylin-phloxine counterstain × 30).

mol for histological examination. Cardiac lesions and nephrocalcinosis were arbitrarily graded (on the basis of macro- and microscopic observations) in terms of a scale of 0 to 3. The means of these findings (with standard errors) and percentual mortality rate are summarized in Table I.

Results. In agreement with our earlier work with intact rats(1), such short pretreatment with Na_2HPO_4 and F-COL or triamcinolone produced neither cardiac necrosis nor nephrocalcinosis. On the other hand, in rats conditioned by the first 2 agents, exposure to any of the 3 stressors resulted in marked cardiac necrosis, usually accompanied by nephrocalcinosis (Figs. 1 and 2), and a mortality rate that reflected severity of organ lesions. Also in agreement with our previous observations on intact rats(1), triamcinolone, a virtually pure glucocorticoid, was much less effective in this respect than F-COL, which possesses both gluco- and mineralocorticoid properties. Interestingly however, occasionally, stressors did produce minor cardiac and renal changes, even in triamcinolone-pretreated animals.

Discussion. Evidently, in themselves ineffective doses of F-COL plus Na_2HPO_4 suffice to sensitize the adrenalectomized rat to production of cardiac necroses and nephrocalcinosis by 3 essentially different nonspecific stressors. Therefore, these actions of stress cannot be relayed through discharge of corticoids or catecholamines, of adrenal origin. This does not mean that production of cardiac necrosis and nephrocalcinosis by stress is independent of adrenal hormones, since exogenous corticoids were given as part of the conditioning procedure and the possibility of discharge of endogenous catecholamines from extra-adrenal sources has not been excluded. Yet, our findings do prove that, here, the corticoids only play a conditioning role and that this, like so many effects of stress, does not depend upon stimulation of endogenous adrenal hormone production. Our findings also confirm that corticoids that exhibit no clear-cut mineralocorticoid effects (*e.g.,* triamcinolone) possess little or no conditioning ability as regards production of cardiac necrosis or nephrocalcinosis, under these circumstances.

TABLE I. Production of Cardiac Necroses and Nephrocalcinosis by Stress in 8 Groups of Adrenalectomized Rats.

Treatment*	Cardiac necrosis (scale 0-3)	Nephrocalcinosis (scale 0-3)	Mortality (%)
F-COL	.0	.0	0
" + cold	1.3 ± .45	.0	10
" + motor denervation	2.7 ± .20	1.4 ± .30	70
" + restraint	2.3 ± .45	1.1 ± .30	90
Triamcinolone	.0	.0	0
" + cold	.0	.0	0
" + motor denervation	.0	.1 ± .10	30
" + restraint	.6 ± .30	.3 ± .20	0

* In addition to treatments listed in this column, all animals were adrenalectomized and given Na_2HPO_4 by stomach tube, as indicated in text.

Summary. In response to various stressors (cold, denervation, restraint), bilaterally adrenalectomized rats, suitably conditioned by pretreatment with certain corticoids and Na_2HPO_4, develop severe cardiac necroses and nephrocalcinosis. Apparently, these manifestations of stress are not mediated through increased secretion of adrenal hormones.

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Effect of Sodium Ferrocyanide and Bicarbonate on Reabsorption of Inorganic Sulfate in Renal Tubules.* (25730)

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Inorganic sulfate is reabsorbed by renal tubules in the dog by a mechanism exhibiting a transfer maximum (Tm)(7). Reabsorption is sensitive to a number of inorganic anions, such as chloride(2), phosphate and nitrate(6) and thiosulfate(1). This paper reports effects of sodium ferrocyanide and bicarbonate. The ferrocyanide ion was chosen because it is not reabsorbed in tubules in the dog(4). Bicarbonate was studied because of its marked effect on phosphate reabsorption (9).

Methods. Experiments were performed on unanesthetized female dogs in supine position. Suitable blood levels were obtained by intravenous priming injections and sustaining infusions. In each experiment, sulfate reabsorption was measured during 3-4 control periods, then in 3-5 periods during infusions of sodium ferrocyanide or bicarbonate. Filtered sulfate was always above Tm loads. In sodium bicarbonate experiments, arterial blood samples were collected anaerobically under paraffin oil. Venous blood samples were used in other experiments. Creatinine was analyzed by method of Bonsnes and Taussky(5), inorganic sulfate by method of Power and Wakefield (11) and bicarbonate according to Van Slyke and Neill(12). Ferrocyanide ion was determined colorimetrically as ferric ferrocyanide (Prussian blue) on trichloroacetic acid (10%) filtrates of plasma and urine, containing 0.1-0.3 μmoles Fe $(CN)_6^{-4}$/ml. 2 ml filtrate + 2 ml $Fe_2(SO_4)_3$-reagent (1 part 17% $Fe_2(SO_4)_3$ + 200 parts saturated solution of gum ghatti). After 20 minutes optical density was read at wavelength 660 mμ. In one ex-

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TABLE I. Effect of Sodium Ferrocyanide on Sulfate Reabsorption. Exp. 2. Dog: M (19 kg).

Time, min.	Urine flow, ml/min.	GFR, ml/min.	$Fe(CN)_6^{-4}$ plasma, μmoles/ml	Sulfate: Plasma, μmoles/ml	Sulfate: Urine, μmoles/min.	Sulfate: Reabsorbed, μmoles/100 ml GF
0	600 ml H_2O orally					
32	Prime 1: 4 g creatinine, 1.5 g Na_2SO_4. Start infusion 2.4 ml/min., 2.4% creatinine, 1% Na_2SO_4.					
79– 85	7.5	70		3.88	118	218
85– 91	8.1	71		3.80	123	206
91– 97	7.5	70		3.98	124	219
97–103	7.5	70		3.94	127	212
107	Prime 2: 7.5 g $Na_4Fe(CN)_6$. Start infusion 7.8 ml/min., 4% creatinine, 3% Na_2SO_4, 2.9% $Na_4Fe(CN)_6$.					
137–143	6.3	84	3.05	4.16	166	219
143–149	6.9	91	3.30	4.32	184	230
149–155	7.0	88	3.34	4.72	174	273
155–161	7.1	90	3.46	4.56	191	242

GFR = Glomerular filtration rate. GF = Glomerular filtrate.

periment, ferrocyanide was analyzed by method of Berliner *et al.*(4). Both sulfate and ferrocyanide precipitate with benzidine. Ferrocyanide was therefore removed by precipitation with *equivalent* amount of zinc acetate before analysis of sulfate. For plasma, 0.01 M zinc acetate in 15% trichloroacetic acid was used, and an additional volume of the acid was added to yield a 3:1 plasma filtrate. Excess zinc must be avoided, since it facilitates precipitation of phosphate with benzidine, thereby giving too high sulfate values.

TABLE II. Effect of Sodium Ferrocyanide on Sulfate Reabsorption in 3 Experiments in Dogs.

Plasma $Fe(CN)_6$, μmoles/ml	Sulfate reabsorption: Control μmoles/100 ml GF	Sulfate reabsorption: $Fe(CN)_6$ μmoles/100 ml GF
4.1–4.9	164	155
5.2–6.2	159	139
3.0–3.5	214	241

Results. Sodium ferrocyanide was given to 3 dogs in 3 experiments (Table I). Table II summarizes the 3 experiments with regard to reabsorption of sulfate before and after administration of sodium ferrocyanide, and ferrocyanide concentrations in plasma. There was no consistent change in reabsorption of sulfate.

Sodium bicarbonate was given to 4 dogs in 10 experiments. Table III shows a typical experiment. The 10 experiments are summarized in Table IV. Administration of sodium bicarbonate depressed reabsorption of sulfate in 9 experiments. The depression averaged 7%; it was not proportional to dose or

TABLE III. Effect of Sodium Bicarbonate on Sulfate Reabsorption. Exp. 15. Dog: E (23 kg).

Time, min.	Urine flow, ml/min.	GFR, ml/min.	Total CO_2 plasma, μmoles/ml	Sulfate: Plasma, μmoles/ml	Sulfate: Urine, μmoles/min.	Sulfate: Reabsorbed, μmoles/100 ml GF
0	700 ml H_2O orally					
12	Prime 1: 2 g creatinine, 1.5 g Na_2SO_4. Start infusion 3 ml/min., 1% creatinine, 8% Na_2SO_4.					
41–51	1.2	98	21.4	3.18	152	164
51–61	"	100	"	3.22	153	168
61–71	"	89	"	3.14	139	157
74	Prime 2: 2 g $NaHCO_3$. 3% $NaHCO_3$ added to infusion.					
80– 90	3.9	97	24.5	3.10	142	163
90–100	6.5	104	24.7	3.02	160	148
100–110	7.7	103	25.9	2.99	160	144
110–120	5.5	100	26.4	3.08	152	155
120–130	4.6	104	"	3.17	163	159

TABLE IV. Effect of Sodium Bicarbonate on Sulfate Reabsorption in 4 Dogs.

Total (CO_2)		Sulfate reabsorption		
Plasma, mmoles/l	Urine, mmoles/min.	Control μmoles/100 ml GF	$NaHCO_3$	% change
26–27	.03– .09	148	134	– 9
23–29	.01– .20	182	161	–12
24–28	.03– .20	155	158	+ 2
24–26	.1 – .2	163	156	– 4
27–28	.3	173	151	–13
22–29	.2 – .4	137	129	– 6
29–35	.3 – .7	194	191	– 2
29–36	.6 –1.2	118	112	– 5
35–43	.7 –1.7	148	138	– 7
37–40	1.3 –1.7	105	87	–17
Avg control				–7%
21–23	<.02			P <.005

plasma level of bicarbonate, and independent of time.

Discussion. According to their effect on reabsorption of sulfate, inorganic anions can be divided into 3 groups: 1. No effect or minor depression: ferrocyanide and bicarbonate ions; 2. Marked depression, immediate effect: thiosulfate(1), acetoacetate, nitrate and phosphate ions(6). 3. Marked depression, 30-50 minutes delay in onset: chloride (2).

Of the 2 ions in first group, ferrocyanide is neither reabsorbed nor secreted in kidneys of the dog, and probably never enters the tubule cells. Bicarbonate, in contrast, is reabsorbed by the tubules. Reabsorption of bicarbonate in distal tubules involves exchange of hydrogen ions for sodium ions(10). The bicarbonate in urine is then converted to carbonic acid, and CO_2 and water diffuse back across the tubular epithelium. The same mechanism probably accounts for reabsorption of bicarbonate in proximal tubules(3). It is therefore possible that no bicarbonate ions enter tubule cells from urine.

The minute effect of bicarbonate on sulfate reabsorption is of doubtful physiological significance, and cannot compare with the effect on phosphate reabsorption (30% depression) (9), which might be due to change in ratio between $H_2PO_4^-$ and $HPO_4^=$ (pK^2 of phosphoric acid = 7.12) in tubular urine, when pH is raised(8).

All anions which depress reabsorption of sulfate (Groups 2 and 3) are themselves reabsorbed in renal tubules. Maximum reabsorption rates of nitrate, acetoacetate or chloride are more than 3 times as high as sulfate Tm(6), but phosphate(6) and thiosulfate(1) have lower Tm than sulfate. Evidently the depressing action of these ions is not merely related to their turn-over rate in tubule cells, but also to their affinity to sulfate transport system.

Summary. Sodium ferrocyanide and sodium bicarbonate have almost no effect on reabsorption of sulfate in renal tubules in the dog. These results, together with earlier studies, suggest that only anions which are reabsorbed as such through tubule cells, depress reabsorption of sulfate. This assumes, however, that bicarbonate is reabsorbed only as CO_2 and water, and not in ionic form.

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Solubility of Human Gallstones in Primate Gallbladder.* (25731)

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Since human gallstones are quite different from those found in animals in regard to high cholesterol content, and since they are dissolved when placed in the gallbladder of such animals as the dog(1), pig, sheep and goat (2), the work on experimental animals cannot be freely translated to man. The only significant difference between bile of man and of experimental animals is found in degree of saturation with cholesterol, *i.e.*, human bile is highly saturated and others are not(3). Since it is not feasible to use human subjects in some of our studies, our effort has been concentrated on finding an animal species in which bile is completely or almost completely saturated with cholesterol as in man(4). Primates aroused our interest because of other similarities to man, and because of their use in studying cholesterol metabolism in relation to atherosclerosis. The present communication demonstrates that human gallstones are less soluble in monkey gallbladder than in other experimental animals, suggesting its bile is more saturated with cholesterol than that of other animals. Comparison of bile constituents of man, monkey and dog is presented together with rate of solution of gallstones placed in the gallbladder.

Methods and materials. Weight of monkeys used in this investigation is about 3.5 lb for Cebus monkey (*Cebus capuchinus*) and 7 to 8 lb for Rhesus monkey (*Macaca mulatta*). They were fed standard monkey chow diet. Under pentobarbital anesthesia the gallbladder was opened by small incision in the fundus. A human gallstone, weighing 100 to 600 mg was inserted into the gallbladder and the incision closed with fine cotton suture in double layers. Care was taken not to pass the suture through the mucosa so as not to cause leakage of bile. The stones used were multiple cholesterol stones containing over 98% of cholesterol obtained from the same gallbladder. After varying lengths of time gallbladders were reopened, the stones recovered and weighed after brief drying in air. Normal human hepatic bile was obtained from choledochostomy tube of a case operated upon after abdominal trauma and pooled. Human gallbladder bile samples were obtained from several patients suffering from diseases other than that of the hepatobiliary system during laparotomy. Several bile constituents, such as bile salts, cholesterol and phospholipids were analyzed, using methods described previously(4).

Results. Dissolution of human gallstones in monkey gallbladder. Eight Rhesus monkeys and 6 Cebus monkeys were used. The results are summarized in Table I, together with dissolution rate of stones in other experimental animals. Human gallstones are much less soluble when placed in monkey gallbladder than in those of other experimental animals, such as dog, hog, sheep and goat. In one out of 8 Rhesus monkeys and 3 out of 6 Cebus monkeys it was found, during second operation, that the cystic duct was apparently obstructed by the stone previously inserted in gallbladder and no bile was found in the gallbladder. Consequently, no decrease of weight of stone was expected in these cases.

Comparison of major constituents of bile from man, monkey and dog. The results of analysis of monkey bile in comparison with those from man and dog are summarized in Table II. There is no fundamental difference in composition between human, monkey and dog bile, except that human and monkey bile have much higher cholesterol concentration.

Identification of bile salts in monkey bile by paper chromatography. Bile salts were isolated from bile using countercurrent distribution according to method of Wiggins and Wootton(6). After alkaline hydrolysis of isolated bile salts, they were subjected to paper partition chromatography according to

* Supported by research grants from Nat. Inst. Health, Research Corp. of Detroit Receiving Hospital, and Parke, Davis and Co.

TABLE I. Solution of Human Gallstones Placed in Gallbladders of Primate and of Usual Experimental Animals.

Animal	Days elapsed	Original wt of inserted gallstone, mg	Wt loss	
			In mg	In %
Experimental animals				
Dog 1	30	1,204.4	364.4	33.0
2	100	1,217.9	756.6	62.2
*Hog 1	37	155	37	23
2	94	159	94	59
*Sheep 1	114	834	527	63
2	142	888	852	96
*Goat 1	72	890	604	68
Primates				
Rhesus monkey 1	60	676.0	16.0	2.5
2	61	333.0	7.9	2.4
3	62	312.0	10.8	3.5
4	92	260.8	8.9	3.4
5	122	53.7	gone†	
6	151	48.8	" †	
7	183	241.8	0 ‡	
8	183	240.1	14.4	6.0
Cebus monkey 1	29	79.6	5.6	6.7
2	30	125.5	0 ‡	
3	31	204.4	25.8	12.6
4	100	139.1	0 ‡	
5	145	216.2	0	
6	145	220.6	8.5	3.9

* Data from Lutton and Large(2).
† Small stones may have been passed.
‡ Stone obstructed cystic duct.

Sjoval(7). Haslewood and Wootton isolated cholic acid from Rhesus monkey bile(8). However, in the present experiment, in addition to cholic acid, paper chromatogram showed presence of chenodeoxycholic and deoxycholic acids in Rhesus monkey bile. In Cebus monkey bile, cholic and chenodeoxycholic acids were present. The presence of a small but definite spot, which has an Rf value between cholic and chenodeoxycholic acids, has been demonstrated on paper chromatogram of bile acids from both kinds of monkeys studied.

Discussion. Although many studies on cholesterol metabolism have been carried out using usual experimental animals, it is not usually possible to assume that these hold in man(9). Gallstones which consist primarily of cholesterol are a definite case in point. We have never found gallstones rich in cholesterol in animals other than man, and furthermore, human gallstones are dissolved when placed in the gallbladder of other experimental ani-

TABLE II. Comparison of Major Constituents of Bile from Man, Monkey and Dog.

	No. of samples	Cholesterol, mg %	Phospholipids, mg/ml	Bile acids — mg/ml			
				Cholic	Deoxycholic	Chenodeoxycholic	Total bile acid
Human							
Hepatic bile	3	113.8 ± 22.9	8.11 ± 1.43	14.1 ± 1.25	1.19 ± .13	2.9 ± .25	18.2 ± 1.84
Gallbladder bile pooled		690.0		26.9	5.6	16.0	48.5
Cebus monkey							
Hepatic bile	3	45.1 ± 23.4	17.3 ± 1.60	3.55 ± 3.56	.15 ± .087*	.58 ± .086	4.28 ± 3.71
Gallbladder bile	7	207.0 ±106.3	26.07 ±15.1	36.6 ±16.3	2.28 ± .60	12.26 ±6.70	51.24 ±20.6
Rhesus monkey							
Hepatic bile	2	49.8 ± 8.2	4.55 ± .67	4.8 ± 1.4	1.6 ± .25	1.1 ± .0	7.4 ± 1.6
Gallbladder bile	2	202.4 ± 82.6	13.20 ± 1.70	28.6 ± 1.2	8.6 ± .4	5.0 ± .2	42.2 ± 1.4
Dog							
Hepatic bile	4	26.3 ± 11.8	13.96 ± 4.27	21.6 ± 6.28	3.29 ±2.22	2.05 ± .32	26.89 ± 8.63
Gallbladder bile pooled		70.0		6.75	15.2	6.4	89.1

* Value for deoxycholic acid is influenced by presence of large amounts of other bile acids(5). All values expressed as mean ± S.D.

mals(1,2), as bile of usual experimental animals can hold more cholesterol in solution than is normally present. On the other hand, human bile is more saturated with cholesterol (3). In our experiment we found that human gallstones are much less soluble in monkeys than in usual experimental animals (Table I), suggesting that monkey bile is more saturated with cholesterol.

There have been few reports indicating incidence of gallstones in monkeys(10). However, it is doubtful that gallstones in monkeys are identical to those of man, which contain a large amount of cholesterol. Lapin and Yakovlava(11) stated they found coagulated gall in the form of cholesterin pigmented clots or cholesterol sand. Fox(12) reported finding pigmented gallstones in the pig-tailed monkey (*Macacus nemestrinus*) but did not analyze the stone. Thudichum(13) stated that gallstones of monkeys are similar to those of man, but he also included pigs, which are now known to have stones of lithocholic acid (14).

Summary. 1. Human gallstones placed in monkey gallbladders are less soluble than those placed in other experimental animals. 2. The major constituents in monkey bile are compared with those in human and dog bile.

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Routine Development of Permanent Strains of Fibroblasts from Bone Marrow of Adult Rabbits. (25732)

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Permanent cell lines of fibroblasts have been established *in vitro* from fetal and adult rabbit tissues, but development of these lines was accomplished only after many failures (1). During studies designed to develop methods for continuous growth and maturation of rabbit bone marrow cells *in vitro* permanent strains of rapidly multiplying fibroblasts could be routinely established from explants of adult rabbit bone marrow. This report describes the relatively simple method used for routine development of permanent strains of fibroblasts from rabbit bone marrow and some growth and morphologic characteristics by these cell lines.

Material and methods. Six-week to one-year-old rabbits were anesthetized with nembutal and a femur was resected. The femur was grasped between 2 large hemostats and cracked with a twisting motion and marrow was carefully removed with No. 11 Bard Parker blade attached to No. 7 holder and placed in a 50 mm Petri dish in 5 ml of culture medium composed of 1 part calf serum, 2 parts medium 199(2) and 0.1% yeast extract (Difco). 400 Units of penicillin/ml and 80 μg/ml of streptomycin were routinely

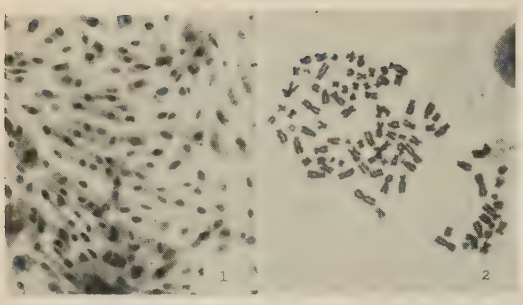

FIG. 1. Rabbit bone marrow strain RBM-1-G fibroblasts, 4 yr in continuous culture. Lillie fixation H. and E., ×50.

FIG. 2. Highly aneuploid RBM-1-G cell with 95 chromosomes, one yr after explantation. Hypotonic medium spread—Acetic orcein, ×800.

added to the medium. The marrow was pumped 4 or 5 times through a 20 gauge needle attached to 5 ml syringe; final suspension contained many small clumps of bone marrow particles as well as isolated bone marrow and peripheral blood cells. 0.3 ml of bone marrow suspension and 5 ml of growth medium were added to each of a series of 12 to 15 T-30 culture flasks, sealed with rubber stoppers and incubated at 36°C. The cells were subcultured by trypsinizing with a 0.05% solution of crystallized trypsin (Worthington Biochemical) in medium 199. When detailed cytologic studies were made, cultures were explanted on standard 3 x 1 inch microscope slides and grown in 8 ml of culture medium in rubber stoppered 6 oz square french bottles.

Results. Most small particles of bone marrow attached to the surface of T-30 flasks within 24 hours. Within 72 to 96 hours after cultures were made a dense outgrowth of spindle shaped cells developed from attached marrow particles. These spindle cells (Fig. 1) multiplied rapidly, so that at end of 10 to 14 days there was a monolayer of fibroblasts covering entire surface of flask. Ten to 15 minutes incubation in trypsin solution was needed to remove cells from the glass at the first and second subcultures, but thereafter they came loose within 5 minutes incubation at 36°C, as quickly as HeLa cells. Cells were subcultured at 5 to 7 day intervals with 2-3 x 10^5 cells added to each new T-30 flask during first 12 weeks of *in vitro* culture. As long as this number of cells was added at each subculture and pH of medium maintained between 6.9 and 7.6, by daily refeeding if necessary, there was no period when cell multiplication stopped. Although rate of multiplication declined during lag period which developed between 10th and 12th weeks, there was very little cellular degeneration. Towards end of lag phase, which lasted about 2 weeks, mitotic figures appeared in many areas of flasks, and this again resulted in formation of a dense monolayer of fibroblasts. After the lag phase, cell strains multiplied with average generation time of about 28 hours and were subcultured in T-30 flasks with 2-5 x 10^4 cells.

Rabbit bone marrow lines were maintained in continuous culture for over 4½ years and during this time fibroblast cells have maintained their spindle shaped morphology.

During the first 6-7 months of growth *in vitro,* there were many cells which contained the normal diploid (44) or near diploid number of chromosomes. After one year *in vitro,* no mitotic figures were observed which had the normal chromosome number and in addition new chromosome types had developed (Fig. 2).

Cells from cultures of fibroblasts derived from rabbit bone marrow have been preserved by both slow and rapid freezing and stored at –76°C in growth media containing 10% glycerol(3). These cells tolerate both slow and rapid freezing equally well with recovery of over 90% of cells originally frozen. Cells frozen rapidly showed an initial lag of 3 or 4 days before they began to multiply rapidly, while those frozen slowly began to multiply 24 hr after they were reexplanted. These rabbit cells tolerate rapid freezing better than those of human or murine origin grown under similar conditions.

Discussion. Permanent lines of cells established by Berman *et al.* from human bone marrow were reported to change in morphology from "fibroblast-like" to "epithelium-like" form during cultivation *in vitro*(4). This morphologic transformation did not occur during establishment of 13 lines of fibroblasts derived from rabbit bone marrow. Cell lines carried in continuous culture for almost 5 years have retained their spindle shaped appearance.

Similar strains of fibroblasts have been established from adult rabbit, spleen, testis and ovary. Epithelial strains have also been isolated from liver and ovary and other tissues containing epithelial cells but not from bone marrow. Epithelial strains were isolated from tissues explanted in plasma clot cultures.

Haff and Swim(1) found that 199 did not support growth of rabbit fibroblast lines. In this laboratory the combination of 199, calf serum and yeast extract has proved an ideal medium for rapid and continuous growth of rabbit fibroblast strains developed from a variety of adult rabbit tissues. In preliminary experiments it was observed that addition of yeast extract increased growth rate and also shortened the lag phase. However, once the lag period was overcome, cell lines derived from marrow and other rabbit tissues proliferate rapidly without yeast extract.

Routine development of fibroblast strains is making possible investigations into possible conversion of normal rabbit fibroblast cells into malignant ones *in vitro*. It is now possible to use the same rabbit for donor tissue and as a recipient after its cells have been grown for any given length of time *in vitro*. The relatively long life span of the rabbit (5 years) makes it another important laboratory animal in which to study mechanisms involved in conversion of normal into malignant cells *in vitro*.

Summary. A simple method is described for routine development of permanent strains of fibroblasts from adult rabbit bone marrow. Marrow particles were grown on glass in medium composed of 1 part calf serum, 2 parts 199 and 0.1% yeast extract. This method is also applicable to development of permanent cell lines from a variety of adult rabbit tissues.

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Non-Specificity of Pituitary-Induced Anuran Ovulation *in vitro*.* (25733)

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Ovulation in *Rana pipiens* induced by injection or implantation of frog pituitary glands is a common laboratory procedure. Implantations or extracts of pituitary glands of a few other vertebrates are also effective in *Rana pipiens, i.e.,* garpike(1), toad(2), newt(3), chicken(4), and sheep(5). Some other anurans, particularly *Xenopus laevis* and several urodeles, ovulate upon exposure to pituitary factors from a wider variety of animals or even pregnant mares' serum and human chorionic gonadotropin(6). In many instances, however, enormous dosages of heteroplastic pituitary tissue are required, suggesting that responses obtained may not be entirely physiological, or that injected material may have merely activated recipient's pituitary gland. Induction of ovulation *in vitro* by addition of extraspecific pituitary factors to Ringer's solution containing ovarian tissue from normal, healthy frogs, rules out participation by the host's pituitary gland, thus providing a very critical test. Routine induction of ovulation *in vitro* with frog pituitary extracts and/or preparations of FSH and LH from sheep pituitary glands has been reported(5,7). It now seems important to record ovulatory responses *in vitro* in a number of interspecific combinations of ovarian tissue and pituitary glands, not only to add data for consideration in the species-specificity controversy, but in view of objection of some(8) that ovulation *in vitro* may

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TABLE I. Ovulation Induced *In Vitro* in Ovarian Tissue from Various *Anura* when Exposed to Homoplastic and Heteroplastic Pituitary Extracts.

Ovarian donors	Pituitary donors					
	Rana pipiens	*Rana clamitans*	*Rana catesbiana*	*Rana sylvatica*	*Pseudacris trilineatus*	*Hyla crucifer*
Rana pipiens	+ Jan.-Dec.	+ June 9	+ June 9 Nov. 14	+ Oct. 26	+ Mar. 31	+ Mar. 31
" *clamitans*	+ June 9	+ June 9	+ June 9			
" *catesbiana*	+ June 9	— June 9	+ June 9			
" *sylvatica*	+ Oct. 26 Feb. 18			+ Oct. 26 Feb. 18		
Pseudacris trilineatus	+ Mar. 31			+ Mar. 30	+ Mar. 30 " 31	
Hyla crucifer	+ Mar. 30 " 31			+ Mar. 30	+ Mar. 30 " 31	+ Mar. 30 " 31

+ = positive response; — = negative response.

not be a wholly normal process.

Methods. Specimens of *Rana pipiens* were obtained periodically from collectors in Vermont and Wisconsin. *Hyla crucifer, Pseudacris trilineatus,* and *R. sylvatica* were collected late in fall or at least 2 weeks before onset of breeding in the vicinity of Ann Arbor, Mich. All preceding species go into hibernation with a fully mature ovary and normally breed immediately upon emerging in spring. Fully mature eggs are present in ovaries, from Sept. until mid-April. *R. clamitans* and *catesbiana,* also obtained locally in Michigan, emerge from hibernation with only partially mature ova, considerable increase in ovarian weight occurring between emergence and breeding late in June. These species are suitable for ovulatory experiments, then, only during about the first half of June. Procedure was essentially that previously described (7), *i.e.,* suspension of ovarian fragments on cotton thread in shell vials containing 10 ml Ringer's fluid with added pituitary extract. For some smaller animals a whole ovary was placed in small Petri dish with 20 ml pituitary-Ringer solution. Pituitary dosages were the equivalent of 1/16 of gland/10 ml of fluid, and were made by triturating fresh tissue in small mortar, adding 1 ml of distilled water to lyse any remaining cells, then diluting to desired volume with Ringer's solution. Controls were always assembled for each species of ovarian tissue on given day, and consisted of ovarian fragments or whole ovaries suspended in Ringer's fluid without pituitary extract. None of these controls ever ovulated spontaneously, indicating that in no instance was ovarian tissue already stimulated to point of ovulation by donor's pituitary gland.

Results (Table I) show uniform responsiveness of ovaries used and lack of specificity of pituitary factors among species represented. Percentages of ovulation were approximately as expected, *i.e.,* 60% or more, if breeding season was imminent and somewhat less (15-50%) in the fall. Ovulation in excess of 95% was obtained with *Pseudacris* ovary when stimulated by either *Pseudacris* or *R. pipiens* pituitary extract; *Hyla* ovary shed about 90% of its eggs when exposed to *R. pipiens* pituitary factors but only about 25% when exposed to its own hypophyseal hormones. The failure of *R. catesbiana* ovary to respond to *clamitans* pituitary would seem more than accidental, since the same tissue responded to pituitary extract from *pipiens* and *catesbiana* on the same day. On the other hand, different areas of the same ovary (at least in *pipiens* work) have been unequally responsive, and a particularly resistant section of *catesbiana* ovary may have been selected inadvertently for exposure to

clamitans pituitary extract. Undoubtedly, many factors enter into potential capacity of any ovary to ovulate on being stimulated by pituitary hormones, and without extensive study, a meaningful evaluation is difficult. It is for this reason that individual percentages of ovulation have been omitted from Table I.

It should be noted particularly that *R. pipiens* ovary has now been induced to ovulate *in vitro* during every month of the year. The ovary of a freshly-caught specimen ovulated Aug. 29th, and September ovulation has been induced on several occasions in freshly-collected frogs. Animals kept in forced hibernation in the laboratory may be used routinely from mid-October into early June (no ovulation in Ringer controls) and on 2 occasions ovulation was induced *in vitro* in mid-July from specimens held in cold room since the previous April.

Negative results have been obtained with *R. pipiens* ovary *in vitro* when pituitary extracts from the following animals were added: sexually immature *Ambystoma maculatum* (Nov. 19), 2 sexually mature turtles (*Pseudemys scripta troosti* and *Chrysemys picta,* Mar. 29), and normal and castrated laboratory white rats of both sexes (Dec. and Mar., on several occasions). Human chorionic gonadotropin (Antuitrin-S, Parke-Davis) and combinations of this urinary factor with mammalian FSH (Synapoidin, Schering) failed to induce ovulation either *in vivo* or *in vitro* in repeated assays, nor have they increased significantly the responses obtained with frog pituitary extract. In every instance potential responsiveness of ovarian tissue was verified simultaneously by exposure of other ovarian fragments to *R. pipiens* pituitary extract.

Summary. Ovulation *in vitro* has been induced in a variety of anurans using homoplastic and heteroplastic pituitary extracts, indicating (1) that the reaction as induced in excised ovarian tissue is truly physiological and normal, and (2) that there is a general lack of specificity of pituitary hormones for this response.

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Enzyme Distribution in Human Brain. Lactic and Malic Dehydrogenases.* (25734)

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Studies on distribution of enzyme activity in mammalian brain have been carried out by histochemical and microchemical methods. These technics have proved suitable for detailed study of small portions of the nervous system, *i.e.,* Ammon's horn(1) and limited areas of cerebral cortex(2). There have been no systematic studies of normal enzymatic activity on the human central nervous system. The present work was undertaken to define normal enzyme distribution in human gross material and to serve as a baseline for similar studies of various neuropathological entities. Lactic dehydrogenase and malic dehydrogenase activities were chosen because they offered the possibility of comparing information obtained by this method with histochemical

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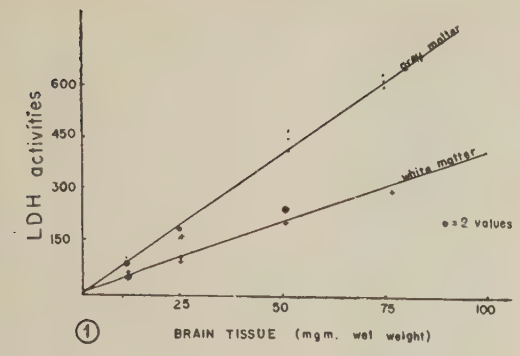
and microchemical data now available. These enzymes are also widespread in their distribution in nervous tissue.

Material and methods. Human brains were obtained from Dept. of Pathology, Peter Bent Brigham Hospital. Fresh material obtained within 3 hours of death was used. Strominger and Lowry(3) have shown, in rabbits, little change in lactic dehydrogenase and malic dehydrogenase activities after death in this time interval. The brain was taken directly from cranial cavity, brought in iced container to the laboratory and then placed in the deep freeze for 24 to 36 hours. Previous experiments had shown this increased the yield of enzymatic activity. This has also been noted by Strominger and Lowry(3). The brain was then thawed at 24°C, and samples between 20-90 mg of tissue were dissected with care to insure homogeneity of tissue (*i.e.,* all gray matter, no blood vessels, tissue from various nuclei, etc.) and immediately weighed. The tissue was then finely ground and chopped in 3 ml of metal-free double distilled water, by use of glass stirring rods. This solution was then allowed to stand for 36 to 48 hours at 24°C, centrifuged at 3000 rpm and the supernatant used for further tests. Both lactic dehydrogenase and malic dehydrogenase activities were measured by disappearance of DPNH measured at 340 mμ using a "Coenzometer." A unit of activity of both malic and lactic dehydrogenase was defined as a change of 0.001 optic density unit/minute/cc of solution. The reaction mixture for lactic dehydrogenase consisted of 1.8 ml metal-free doubled distilled H_2O, 1 ml of 0.1 M PO_4 buffer at pH 7.4 (Mallinckrodt A.R.); 0.1 ml of 0.002 DPNH (Sigma) adjusted to pH 7.4, 0.1 ml of 0.001 M pyruvate (Mathieson, Coleman and Bell) and a 0.1 ml solution of enzyme. All reagents were prepared freshly each day. The reaction was initiated by addition of pyruvate solution and readings were taken over a 3 minute period. Control reactions without addition of substrate showed no measurable decrease in DPNH concentration in 3 minutes. The reaction mixture for malic dehydrogenase consisted of 0.3 ml of 0.2 M Sodium Pyrophosphate (J. T. Baker Chemical Co. analyzed reagent) at pH 8.3, 0.2 ml of 0.002 DPNH (Sigma) prepared as above, 2.3 ml of metal-free distilled H_2, 0.1 ml of enzyme and 0.1 ml of 0.0076 M oxaloacetic acid (H. M. Chemical Co., Ltd). The reaction was initiated by addition of oxaloacetic acid and readings taken for 2 minutes. Control reactions without addition of substrate showed no measurable decrease in DPNH concentration in 3 minutes. Enzyme activities were given/mg wet weight of sample studied. Protein content of gray matter was also roughly estimated by E280 absorption of solution on Beckman spectrophotometer. Values of enzyme activity obtained were confirmatory of enzyme activity as calculated on wet weight basis. Presence in white matter extracts of a slight opalescence interfered with E280 measurements and prevented comparison of white and gray matter extracts. Wet weight was used since it appeared to be a more specific, accurate and reproducible baseline and it allowed comparison. Therefore data are reported on this basis. Purified enzyme solutions of lactic dehydrogenase and malic dehydrogenase obtained from Nutritional Biochemicals Corp. were used as standards. All samples for comparison from different areas were run in one batch to prevent undue variation in time permitted for enzyme extraction. All measurements were made in triplicate on individual samples. Multiple samples were taken from each area.

Results. A number of experiments were performed using H_2O, isotonic saline, PO_4 buffer pH 7.4, and veronal buffer pH 8.6 as extracting medium for LDH in both gray and white matter. H_2O consistently proved to be the most efficient extractor of enzyme activity. When ground up brain tissue was placed in the solvent there usually was a slow increase in LDH enzyme activity over 4-8 hours. From 12 hours to 48 hours most preparations showed constant activity which did not vary more than 10-15%. About 20% of preparations showed a peak activity at 24 hours that was within 20% of the activity measured at 12 hours and 72 hours. After 72 hours all preparations showed a slow decline in activity (when kept at room temperature) which resulted in about 50% activity loss in 10 days.

Extractions were run at 4°, 24°, 37°. Op-



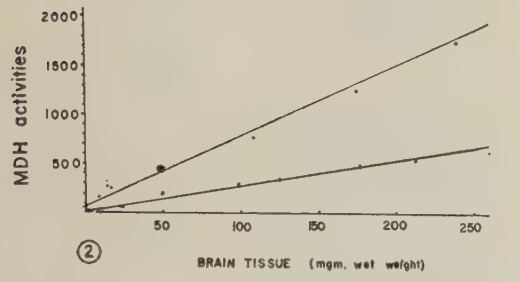


FIG. 1. Relationship of LDH activity to brain extracts.

FIG. 2. Relationship of MDH activity to brain extracts. (Upper curve represents gray matter, lower curve white matter.)

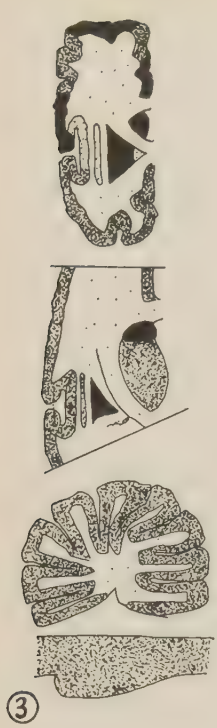
timal extractions occurred at 24° for both white and gray matter. Finely chopped and homogenized material gave roughly the same LDH values on 2 samples. 5.5 ± 1.8 units per mg wet weight for finely chopped brain; 6.0 ± 1.3 units/mg wet weight for homogenized (12 determinations of each). It was therefore elected to use chopped material for the assays reported.

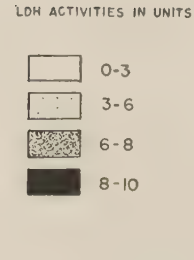
In the range of 0-100 mg of brain tissue/3 cc of solution, there is a linear extraction of enzyme activity. Above 200 mg of tissue there is some decrease in efficiency of extractability of enzyme activity. Both gray matter (cortex) and white matter (centrum ovale) were used in demonstration of linear extraction with increasing amounts of tissue (Fig. 1).

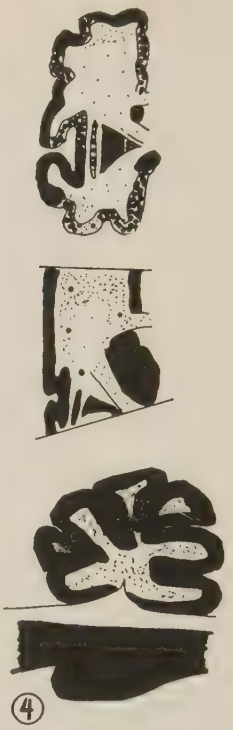
Malic dehydrogenase was extracted with H_2O at room temperature. Analysis of a sample 12 times gave a value of 9.2 ± 2.43 for white matter and 20.4 ± 5.19 for gray matter. In the range of 0-250 mg of both white and gray matter there is a linear extraction of enzyme activity. The range above 250 mg of tissue/3 cc solution was not tested (Fig. 2). When in contact with the extracting solvent malic dehydrogenase activity rapidly increased for the first 6-8 hrs and then stabilized. In one typical experiment there was no significant change from 8 to 82 hrs, then up to 10% rise was gradually noted through 196 hrs.

All data reported here were obtained on material extracted by water at 24°. Since values were reasonably constant from 24-48 hours, no attempt was made exactly to standardize time of activity determinations, but all were performed between 36-48 hours. The most striking result was the clear presence of more enzyme activity in all gray matter as compared to all white matter (Fig. 3 and 4 and Table I for both enzymes). Average value for 43 assays of gray matter for lactic dehydrogenase was 6.1 ± 1.1 units/mg wet weight and for white matter was 3.5 ± 0.6 units (27 assays). A similar finding was noted for malic dehydrogenase with 21.2 ± 4.6 units noted for 39 assays of gray matter and 11.7 ± 2.3 units (21 assays) noted in white matter. In both cases more variation was noted in gray matter samples than in white matter. This suggested the possibility that certain areas of brain might be even more highly concentrated than others. Table II shows a breakdown of the source of gray matter.

Lactic dehydrogenase. The level of enzyme activity extracted from inner part of globus pallidus is more than 2 standard deviations below mean value of all gray matter assays. This probably reflects the great amount of white fibers passing through this nucleus. The caudate and outer part of the globus pallidus show significantly higher extractable enzyme activities than the rest of the gray matter, 8.5 and 9.2 units/mg wet weight respectively. In white matter (Table III), there is less variation. The white tracts of the cerebral hemispheres seem to be reasonably similar in activity. Optic tracts and the septic pellucidum, fornix and cerebellar white matter all tend to run higher than hemispheral values. The olfactory bulb and tract consistently had the lowest lactic dehydrogenase values. Fig. 3 is a graphic representation of lactic dehydrogenase distribution in a typical experiment.







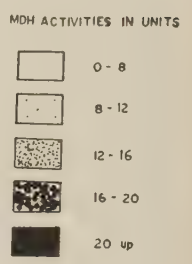


FIG. 3. LDH distribution in brain.
FIG. 4. MDH distribution in brain.

Samples were taken from representative areas as noted and their activity is pictured on the graph. For purpose of illustration activity ranges were used rather than individual values. It can be clearly seen that gray matter values were consistently higher than white matter values. Variations between adjacent activity ranges are probably not significant. Caudate and globus pallidus were consistently among the highest activities in all 3 brains examined.

Malic dehydrogenase. Malic dehydrogenase has a much more uniform distribution in gray matter (Fig. 4) and variation between various anatomical sites is not as striking as in lactic dehydrogenase. Again enzyme activities are plotted as "activity ranges" for purposes of illustration. Changes in adjacent activity ranges are probably not significant. Range in gray matter was 16-23 units/mg wet weight, whereas range in white matter was 10-15 units/mg wet weight. The differences noted with lactic dehydrogenase, *i.e.*, excess of enzyme activity in the optic tracts, septum pellucidum and fornix, and decreased amounts in olfactory bulb and tract are no longer apparent (Table III).

Discussion. Distribution of 2 enzyme activities, extracted under similar conditions from human material, showed certain phenomena in general, as well as individual variations. Both tend to occur predominantly in

TABLE I. Enzyme Distribution in Brain.

	Lactic dehydrogenase activity*		Malic dehydrogenase activity*	
Exp.	Gray matter	White matter	Gray matter	White matter
I	8.1 ± 1.7 (12)	3.8 ± .5 (10)	22.8 ± 6.4 (17)	12.0 ± 2.4 (10)
II	5.3 ± 1.0 (8)	3.5 ± .7 (6)		
III	5.3 ± .9 (23)	3.2 ± .7 (11)	20.0 ± 3.2 (22)	11.4 ± 2.3 (11)
Total	6.1 ± 1.1 (43)	3.5 ± .6 (27)	21.2 ± 4.6 (34)	11.7 ± 2.3 (21)

* MDH and LDH units/mg wet wt. Each exp. was done on different human brain.

TABLE II. Enzyme Distribution in Various Areas of Gray Matter.

	Units/mg wet wt	
	LDH	MDH
Frontal cortex	7.5	22.1
Temporal "	6.1	19.4
Parietal "	6.2	20.3
Occipital "	7.1	19.8
Putamin	6.8	21.2
Caudate	8.5	23.1
Globus pallidum, inner part	3.2	20.3
" " , outer "	9.2	22.0
Claustrum	4.6	20.0
Thalamus	5.9	20.7
Brain stem reticulum	6.8	21.4
Amygdala nucleus	4.5	11.8
Cerebellar cortex	6.2	21.1

gray matter. In individual nuclei lactic dehydrogenase varied independently of malic dehydrogenase, however. It has been suggested that these enzymes are more concentrated in nerve cells and/or synaptic areas(2).

Robbins *et al.*(4) found more malic dehydrogenase and lactic dehydrogenase in cortex than in white matter of rabbit and monkey brain and noted that the malic dehydrogenase/lactic dehydrogenase ratio is slightly higher for cortex than for white matter. We could confirm these observations. They also noted areas where the correlation of enzyme concentration was not parallel. Lactic dehydrogenase showed more variation than malic dehydrogenase in central white matter and optic nerves. Strominger and Lowry(3) previously have noted increase of lactic dehydrogenase in optic nerve and tracts by their microtechnics, and their coworkers have commented on the marked differences noted in histologically different areas of brain, and they have spoken of enzymatically different types of tracts.

TABLE III. Enzyme Distribution in Various Areas of White Matter.

	Units/mg wet wt	
	LDH	MDH
Frontal	3.6	12
Temporal	3.6	12
Occipital	3.5	13
Corpus callosum	3.6	11
Cerebellar white	4.4	15
Optic tracts	4.5	10
Septum pellucidum and fornix system	4.2	10
Olfactory bulb and tract	2.9	13

The higher concentration of lactic dehydrogenase in the caudate nuclei and other central gray areas is of interest in that it correlates with the observations of Himwich and Fazekas(5) that the caudate nucleus has one of the greatest oxygen consumptions found in brain tissue.

We were unable to find significant differences between the various areas of cortex, a finding noted by Bennett *et al.*(6) to be true of cholinesterase. He noted that cholinesterase activity differed in motor and visual somesthetic cortices, but that this was not true of lactic dehydrogenase. Our observations confirm his observations on lactic dehydrogenase and show similar diffuse distribution for malic dehydrogenase.

Summary. Distributions of enzymatic activities of lactic dehydrogenase and malic dehydrogenase have been studied in human brain. Their activities are increased in gray matter as compared to white matter. There are areas where concentrations of these enzymes seem consistently elevated, with higher concentration of lactic dehydrogenase in caudate nuclei, as compared to other gray matter, and optic nerve as compared to other white matter. In some areas lactic dehydrogenase activity shows no correlation with malic dehydrogenase activity. The significance of these variations is not as yet apparent.

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Individual Variability in Relationship of Human Ketosteroid Excretion To Urine Volume.* (25735)

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(Introduced by J. P. Marbarger)
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Numerous investigators(1-3) demonstrated that changes in urine volume do not significantly alter human 17-ketosteroid (17-KS) excretion. Others suggested that both 17-KS (4,5) and corticoid(6,7) excretions are dependent on urine volume. Similar discrepancies have been observed in rabbits(8,9) and guinea pigs(10,11) but not in rats(12) and swine(13). Prior to successful use of urinary steroid output as index of adrenocortical activity at least 4 variables must be considered: 1) urine volume, 2) steroids, 3) individual variability and 4) species differences. This study was undertaken to determine possible relationship of total ketosteroids (TKS), 17-ketogenic steroids (17-KG), 17-ketosteroids (17-KS) and dehydroepiandrosterone (DIA) to urine volume.

Materials and methods. Two experiments were carried out. In the first accurate 24-hour urine specimens were collected by 13 healthy men (20-38 years). Each subject acted as his own control while maintaining a record of daily *ad lib* fluid intake. Subsequently for 3 consecutive days (forced diuresis), subjects drank water at least twice that consumed during control period. Neither control nor experimental days coincided for all subjects, thus minimizing environmental effects. Aliquots of urine were refrigerated at 4°C 2-3 weeks prior to analysis for 17-KS by the method of Vestergaard(14). The second experiment determined spontaneous variability in urine volume and steroid excretion. Ten males (25-36 years), 6 of whom were subjects on first experiment, collected urine specimens for 7 to 34 days. As in previous experiment no attempts were made to control diet or to influence subjects' way of living. They recorded quantities of fluid and solids ingested during normal daily routine. Aliquots of daily urine samples were hydrolyzed and extracted for TKS, 17-KG, and 17-KS by procedure of Norymberski(15), then assayed for ketosteroids by Zimmermann reaction as modified by Holtorff and Koch(16). DIA determinations(17) were performed on aliquots of neutral 17-KS fraction.

Results. Ingestion of large quantities of fluid caused 3-fold increase in urine and mean 17-KS increase of 6% from control mean. Marked increases in urinary 17-KS were found in subjects 5 (34%) and 13 (27%), both had 2 of highest increases in urine. On the other hand maximum depressions of 25% were observed on third day of forced diuresis in subjects 10 and 16. Statistical analyses revealed that group changes in 17-KS excretion for any control day did not differ significantly ($P > 0.5$) from other days, whereas changes in fluid intake and urine volume were highly significant ($P < .001$). Four (2, 5, 8 and 13) of 13 subjects exhibited significant positive correlations, while 6 of the remaining insignificant correlations tended to be slightly negative (Table I). Nevertheless, probability levels of coefficients of correlation within samples ($r = +.42$), between subjects ($r = +.39$) and for total population ($r = +.27$) were obtained at $<.01$, $<.05$, and $<.05$ levels, respectively.

Spontaneous variations in coefficients of correlation between urine volume and ketosteroid excretion are presented in Table I. Urine for individual subjects varied from 0.81 to maximum of 1.99 liters/day, with group range of 0.52 to 3 liters/day. Unlike the forced diuresis experiment, 3 significant negative correlations (Subj. 1, 15, 17) in addition to one significant positive correlation (Subj. 17 for 17-KG) were observed. When all data for a specific group of steroids were combined,

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‡ From thesis submitted in partial fulfillment for degree of M.S.

TABLE I. Individual Variability in Ketosteroid Excretion and Coefficients of Correlations.

Forced diuresis		Daily fluctuations in ketosteroid excretion							
17-KS		Urine vol	17-KS		TKS		17-KG		DIA	
Mean*	r†	range, l/day	Mean	r	Mean	r	Mean	r	Mean	r
28.1 ± 1.7	−.01	1.04-1.85	30.5 ± 1.2	−.63	44.4 ± 2.4	−.80‡	13.9 ± 2.1	−.61	10.4 ± .6	−.52
25.4 ± 2.8	.83‡									
17.8 ± 1.2	−.02									
		1.35-2.56	17.6 ± 1.4	.45	27.9 ± 1.3	.08	10.3 ± 3.4	−.42	3.7 ± .3	−.56
23.4 ± 2.3	.97‡	.52-1.57	21.8 ± 3.4	−.37	31.1 ± 2.5	−.23	9.3 ± 2.5	−.06	5.5 ± .3	−.23
14.2 ± 1.4	−.35									
10.3 ± 1.2	−.47									
18.7 ± 1.4	.82‡	.70-1.54	16.2 ± 1.1	.35	25.7 ± 1.3	−.23	9.5 ± 1.7	.37	4.2 ± .2	.41
12.9 ± .5	.05	.76-2.26	14.8 ± .9	.48	25.6 ± 3.2	.18	10.8 ± 3.	.02	3.5 ± .3	−.05
9.2 ± 1.4	−.02									
23.4 ± 1.6	.50	1.26-2.	24.6 ± 1.5	.05	36.4 ± 1.5	.15	11.8 ± 1.5	.14	2.6 ± .2	.08
11.2 ± .9	.04									
29.3 ± 2.5	.96‡	.56-2.34	28.7 ± 2.3	.47	39.2 ± 1.4	.06	10.5 ± 2.4	.41	7.9 ± .3	.30
		1.09-3.	15.5 ± 1.3	−.24	28.5 ± 1.5	−.09	13.1 ± 1.9	.18	2.8 ± .3	.43
		1.12-2.	20.4 ± 2.4	.39	32.0 ± 2.2	−.27	11.6 ± 1.7	−.91‡	5.1 ± .5	.46
12. ± 1.7	−.14									
		.70-1.68	13.9 ± .9	−.33	26.9 ± 2.2	.22	13.1 ± 2.6	.35‡	4.9 ± .5	−.47‡

* mg/24 hr ± S.D. † Coefficient of correlation. ‡ P .05.

only 17-KS excretion appeared dependent on urine volume ($r = +.49$; $P < .05$).

In 3 (1, 9, 11) of 6 subjects who participated in both experiments, 17-KS excretion was not related to urine volume in either experiment; the remaining 3 subjects (5, 8, 13) although exhibiting significant positive correlations during forced diuresis, showed no such correlation in the second experiment.

Discussion. Twenty-four hour excretions of TKS, 17-KS, 17-KG and DIA when correlated with subject's 24-hour urine volume varied not only from individual to individual, but also from time to time in the same individual. Of 53 coefficients of correlation, 5 were significantly positive (4 for 17-KS; 1 for 17-KG) and 3 were significantly negative (1 for TKS; 1 for 17-KG; 1 for DIA). The latter indicate that in some individuals increased urine volume may be associated with marked depression in ketosteroid output. However in most cases excretion of these steroids remained relatively constant although urine volume varied by a liter or more.

Diuresis, as a result of forced fluid intake, had a variable effect on individual urinary 17-KS excretion; in some instances it increased, in others remained relatively constant, and in a few decreased. All 4 positive 17-KS correlations were observed during forced diuresis. Since 3 subjects showed no correlation when placed on second experiment, forced fluid intake may have imposed some stress. It is apparent that when studying adrenocortical function results may be dependent on type and number of subjects. This was further demonstrated when data for all subjects in the second experiment were combined. A significant positive correlation is observed only for the 17-KS fraction. However in view of variability in individual coefficients of correlation these data suggest that subjects who normally excrete large quantities of urine tend to be high 17-KS excretors and *vice versa*; but it does not necessarily follow that titers of 17-KS are dependent upon fluctuations in urine within the same individual.

Discrepancies in the literature relative to effect of urine volume on ketosteroid excretion in normal humans may be the result of: 1) stress response to severe hydration or dehydration to give wide variations in urine; 2) limited number of subjects; 3) neglect of individual variability, and 4) grouping of all subjects' data for coefficients of correlation. Our experiments indicate that variations in urine as a result of relatively large fluctuations in daily fluid intake do not significantly alter excretion of ketosteroids except in some cases where stressful factors (forced diuresis, etc.) are superimposed. Whether this also holds true for other species awaits further experimentation.

Summary. Concentrations of total keto-

steroids, 17-ketosteroids, 17-ketogenic steroids, and dehydroepiandrosterone were determined in urine collected from healthy young men who ingested various quantities of fluid. Individual coefficients of correlation between urine and ketosteroid output varied from significantly positive to negative, with the majority showing no correlation. These results suggest that in most men daily variations in urine volume do not influence excretion of ketosteroids. However, group coefficients of correlation reveal that a significant positive correlation was obtained only for 17-ketosteroids. These data suggest that 1) men who normally produce relatively large volumes of urine also excrete large quantities of this steroid, and 2) type and number of subjects must be considered when calculating group coefficients of correlation.

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Effect of Co^{++}, Ni^{++}, and Zn^{++} on Corticoid Excretion by the Guinea Pig.* (25736)

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The guinea pig is useful for study of factors which influence ACTH release(1,2,3) since urinary corticoids are readily measurable in this species. The effect of Co^{++} was investigated because it has been suggested that events leading to establishment of polycythemia by this substance follow from production of a relatively anoxic state(4). It was observed that increased corticoid excretion followed intraperitoneal (but not subcutaneous or intramuscular) administration of Co^{++}. Increased corticoid excretion was also induced by intraperitoneal injection of Ni^{++} and Zn^{++}.

Methods. The salts employed were $FeCl_2 \cdot 4H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $HgCl_2$, $ZnCl_2$, $PbCl_2$, $CuCl_2 \cdot 2H_2O$. 300-350 g animals of either sex were injected intraperitoneally with various divalent ions, in 5 ml of saline; control animals received 5 ml saline as well. Co^{++} was also administered along with 2.5 mg morphine sulfate/100 g according to procedure previously described(2). Urine was collected for 6 hours(3). Urinary corticoids were determined by methods of Silber and Porter(5). When Co^{++} was administered subcutaneously or intramuscularly it was administered in 0.25 ml saline. To compare toxicity of these ions Co^{++}, Ni^{++}, Fe^{++}, Cu^{++} and Zn^{++} were administered twice daily at

* Supported by grant from U.S.P.H.S.
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TABLE I. Effect of Divalent Ions on Urinary Corticoid Excretion. 4 μM/100 g. 6 hr collection period.

Group	No.	Corticoids	Group	No.	Corticoids
A. *Subcut. route*			C. *Intraper. route*		
Control	6	127 ± 19*	Control	18	103 ± 14
Co^{++}	6	112 ± 28	Co^{++}	11	204 ± 31§
B. *Intramusc. route*			Ni^{++}	18	190 ± 25§
Control	6	108 ± 17	Fe^{++}	12	109 ± 12
Co^{++}	6	121 ± 20	Fe^{++}†	18	141 ± 14
			Cu^{++}	18	112 ± 22
			Pb^{++}	18	118 ± 17
			Zn^{++}	18	199 ± 28§
			Hg^{++}‡	14	90 ± 19

* Stand. error.
† 16 μM/100 g.
‡ 2 μM/100 g, when 4 μM/100 g was administered, all animals died while in collection cages.
§ p value $<.01$.

doses of 4 μM/100 g to each of 10 animals.

Results. Co^{++} administered intraperitoneally (but not subcutaneously or intramuscularly) induced an increased urinary corticoid excretion. It was determined with use of graded doses of Co^{++}, that a dose of 4 μM/100 g consistently doubled the 6-hour excretion value. Accordingly, this dose was employed for other ions (Table I). The response to Co^{++}, Ni^{++} and Zn^{++} was similar. Cu^{++} and Pb^{++} induced a moderate, but insignificant increase in corticoid excretion (1.1-1.2 ×) Fe^{++} did not produce any effect although 40% increase was observed if dose was increased 4-fold. Hg^{++} at this dose level proved lethal. When administered at dose level of 2 μM/100 g (non-fatal) corticoid excretion was 10% below controls. Diarrhea was present in all but one of animals which received this dose of Hg^{++}. Several animals which received Cu^{++} died after collection was completed. No toxicity was manifest with other ions following a single injection. A red pigment was observed in urine of Co^{++} treated animals but not in others.

In the toxicity study Ni^{++} and Cu^{++} caused death of all within 5 days. At this time only one animal receiving Co^{++}, 2 receiving Fe^{++} and 3 receiving Zn^{++} had died. After 8 days, 5 receiving Co^{++}, and 3 receiving Fe^{++} and Zn^{++} remained. Gross inspection revealed that survivors from Co^{++} had peritonitis with ascites and obvious hepatic and splenic pathology. Fibrinous exudation was present. The peritoneal cavity of Zn^{++} and Fe^{++} treated animals appeared normal except for 2 mm zone of hepatic necrosis in one of the former, and a peritoneal adhesion to liver, in one of the latter. None of the 10 control animals, which received intraperitoneal injections of saline, exhibited any pathology.

Inspection of peritoneal cavity of guinea pigs 6 hours after a single injection of Co^{++} suggested presence of slight hyperemia.

Co^{++} was administered along with morphine to determine whether it acted at some site before or after morphine sensitive areas in the brain, in the central nervous system, hypothalamus, anterior pituitary, adrenal complex, involved in increased adrenal steroid production. There was a marked reduction in adrenal response (Table II).

Discussion. The 3 ions Co^{++}, Ni^{++} and Zn^{++} have approximately the same ability to increase corticoid excretion on an equimolecular basis. There appears to be no other property of these ions, not possessed by one or more of the other ions tested, which might explain these findings. Peritoneal reaction was not observed following Zn^{++}. The red pigment appeared only after Co^{++} administration. Ni^{++}(4) and Zn^{++} are not known to be capable of inducing erythropoiesis. Perusal of the literature in regard to the effects of various ions on enzymatic activity fails to reveal those which are common to Co^{++}, Ni^{++} and Zn^{++}, but not to other ions which failed to cause increased corticoid excretion.

The fact that Co^{++} is not effective in inducing increased corticoid excretion when administered subcutaneously and intravenously at dose of 4 μM/100 g, suggests that for Co^{++}, at least, the site of action may be found in an organ served by the portal system. For example, a relatively large dose of Co^{++} entering the pancreas might induce alpha cell

TABLE II. Effect of Morphine on Corticoid Excretion Following Co^{++} Administration.

Group	No.	Corticoids
Control	11	99 ± 7‡
M*	11	45 ± 4
Co^{++}†	12	203 ± 24
M* + Co^{++}†	12	93 ± 14

* 2.5 mg morphine sulfate/100 g was administered according to(2).
† 4 μM/100 g.
‡ Stand. error.

damage of the pancreatic islet cells(6). Ni^{++}, but not Zn^{++}, acts likewise in the rabbit(7). It is also known that Co^{++}, Ni^{++} and Zn^{++}, but not other ions tested, react with insulin(8). However, it is not clear how these mechanisms would lead to increased corticoid excretion.

Morphine caused a marked reduction in adrenal response to Co^{++} suggesting that it acts prior to morphine sensitive area in the brain. Because of possibility of complex formation between Co^{++} and morphine, interpretation of the finding must be accepted with caution.

Summary. Several divalent ions were administered intraperitoneally to guinea pigs, at a dose of 4 μM/100 g body weight. Urinary corticoid excretion was doubled by Co^{++}, Ni^{++} and Zn^{++}. Fe^{++}, Cu^{++}, Hg^{++}, and Pb^{++}, produced little or no effect upon corticoid excretion.

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Accumulation of Strontium-90 and Calcium-45 by Fresh Water Fishes.* (25737)

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Our previous studies suggested that fresh water fishes do not discriminate against strontium relative to calcium when the isotopes are taken up from water in which they swim(1,2). Unlike fresh water fishes, Boroughs *et al.*(3) found that marine *Tilapia* discriminate against strontium relative to calcium. Discrimination against strontium also appears to be well documented in small laboratory mammals and in man(4-6). However, Wasserman *et al.*(7) found that strontium discrimination in rats is not affected by dietary calcium levels. The present results, using double tracer technics, indicate that fresh water fishes display only a small degree of discrimination against strontium relative to calcium and essentially substantiate our previous data.

Methods and materials. Adult fresh water fishes, obtained from commercial sources, were of mixed sexes for zebra fish (*Danio*) and white cloud mountain fish (*Tanicthys*) but guppies (*Lebistes*) were sexually mature males. Fish were placed in artificial pond water(8) containing both strontium-90 and calcium-45. The basic solution, adjusted to pH 7.0, contained 50 mg/liter of each of the following analytical reagent grade salts: sodium nitrate, potassium sulfate and magnesium sulfate. Solutions were modified by additions of calcium chloride, strontium chloride and sodium chloride as shown in results. Addition of strontium-90 and calcium-45 contributed approximately 0.25 mM/liter calcium(2). Small amounts of these ions in the basic solution are not included in the calculation. After 5 days in various solutions, fish were sacrificed and prepared for radioactive assay as previously described(9). Samples were held for 21 days to attain secular equilibrium of strontium-90 and its yttrium-90 daughter nuclide. Total beta activity (strontium-90 plus calcium-45) was determined with windowless gas flow counter. The

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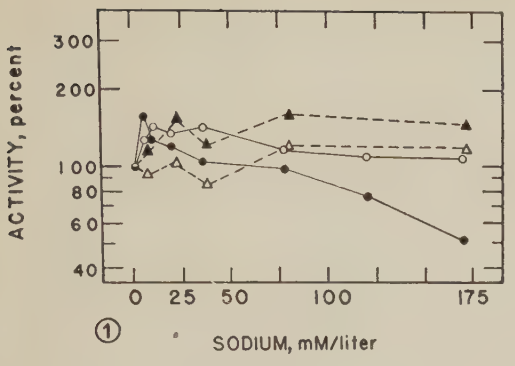

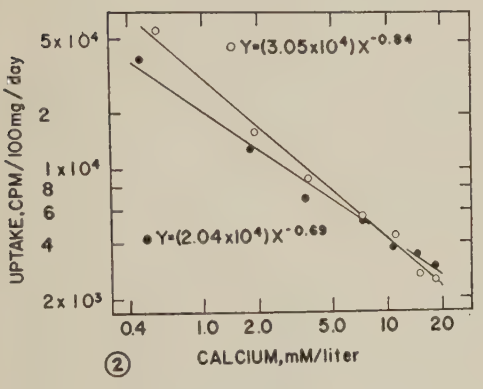

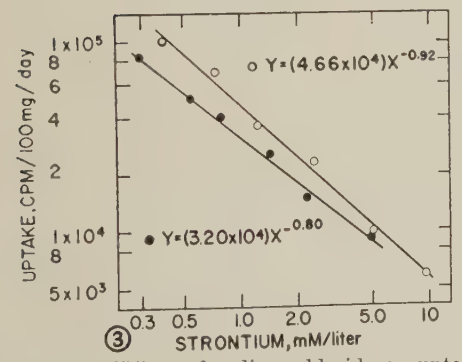

FIG. 1. Effect of sodium chloride on uptake of strontium-90 (▲, ●) and calcium-45 (△, ○) by male *Lebistes* in 2 separate experiments. Water contained approximately 10^5 cpm/ml of each isotope.

FIG. 2. Effect of calcium chloride on uptake of strontium-90 (●) and calcium-45 (○) by male *Lebistes*. Water contained approximately 10^5 cpm/ml of each isotope.

FIG. 3. Effect of strontium chloride on uptake of strontium-90 (○) and calcium-45 (●) by male *Lebistes*. Water contained approximately 10^5 cpm/ml of each isotope.

yttrium-90 component was determined with mica-end window Geiger counter with aluminum absorber (65 mg/cm^2) to absorb the weak beta emissions of calcium-45 and strontium-90. Samples of water were assayed in identical manner. Sample counts were corrected for the absorber, decay and self absorption with previously determined factors.

Results. Two typical experiments obtained with *Lebistes,* demonstrating variable effect of increasing sodium chloride concentration on accumulation of strontium-90 and calcium-45 are shown in Fig. 1. In these and other experiments, an increase in osmotic pressure resulted in either an increase or decrease in accumulation of both strontium-90 and calcium-45. In all instances however, the effect of sodium chloride on strontium-90 accumulation was essentially parallel to that of calcium-45. Similar results were also obtained for other fresh water fishes, *Tanicthys* and *Danio.*

Addition of non-radioactive strontium or calcium chlorides to the basic salt solution depresses accumulation of strontium-90 or calcium-45 in *Lebistes* (Fig. 2,3). Decrease in accumulation of both nuclides with increasing alkaline earth elements is adequately described by a function of the form $Y = aX^b$, and is similar to data previously described for calcium(9). However, the regression lines are not parallel but tend to cross at higher concentrations of salts indicating that strontium ion depresses accumulation of strontium-90 more than calcium-45 and, conversely, calcium ion depresses accumulation of calcium-45 more than it does strontium-90.

We previously showed(1) that accumulation of strontium-90 and calcium-45 by fresh water fish is a function of activity of the water in which they swim. This function, which we called the "concentration factor" (2), is adequately described by the equation,†

$$\text{Concentration Factor} = \frac{\log \text{uptake in cpm/100 mg/day}}{\log \text{water activity in cpm/ml}}.$$

This relationship has recently been confirmed for salt water *Tilapia* by Townsley and colleagues(10). A relationship between concentration factors of one isotope (strontium-

† The "concentration factor" in ref. 2 is erroneously given as $\log \frac{\text{uptake in cpm/100 mg/day}}{\text{water activity in cpm/ml}}$ although the text discussion is accurate.

TABLE I. Effect of Sodium Chloride on Rate of Uptake of Ca^{45} and Sr^{90} and Discrimination of Sr^{90} by Fresh Water Fishes.

Species	Sodium conc., mM/l	Rate of uptake ± S.E. Ca^{45} 10^4 cpm/100 mg/day	Rate of uptake ± S.E. Sr^{90} 10^4 cpm/100 mg/day	Discrimination factor ± S.E.	"t"†	P
Lebistes		9.55 ± .74	16.4 ± 1.76	.949 ± .003 (9)*	16.12	<<.01
Tanicthys	Basic	4.09 ± .34	12.9 ± .75	.921 ± .011 (8)	15.72	"
Danio		.59 ± .07	2.05 ± .20	.964 ± .013 (8)	9.88	<.01
Lebistes		8.89 ± .98	19.3 ± 4.00	.966 ± .005 (8)	7.36	"
Tanicthys	5.75	3.43 ± .21	10.8 ± .64	.920 ± .002(10)	43.91	<<.01
Danio		5.57 ± .50	2.18 ± .16	.984 ± .010 (7)	1.81	>.05
Lebistes		9.68 ± .90	25.4 ± 2.24	.987 ± .010 (9)	1.41	"
Tanicthys	19.5	3.25 ± .15	12.3 ± .81	.936 ± .004 (9)	8.34	<.01
Danio		.50 ± .02	2.22 ± .09	.995 ± .006 (6)	1.05	>.05
Lebistes	172	10.8 ± .68	24.1 ± 2.04	.970 ± .002 (8)	16.81	<<.01
Tanicthys	172	2.19 ± .35	11.8 ± .23	.976 ± .008 (9)	2.98	.02
Danio	120	.25 ± .04	1.17 ± .18	1.034 ± .010 (6)	3.38	"

Water activities: *Lebistes*—$Ca^{45} = 8.71 \times 10^4$ cpm/ml; $Sr^{90} = 2.77 \times 10^5$ cpm/ml. *Tanicthys*—$Ca^{45} = 4.02 \times 10^4$ cpm/ml; $Sr^{90} = 3.54 \times 10^5$ cpm/ml. *Danio*—$Ca^{45} = 4.85 \times 10^3$ cpm/ml; $Sr^{90} = 3.99 \times 10^4$ cpm/ml.

* Values in parentheses indicate No. of fishes analyzed.

† "t" values calculated from differences of concentration factors for Ca^{45} and Sr^{90}. P values indicate probability of discrimination factor being significantly different from 1.0.

90) to another (calcium-45) may be expressed as a "discrimination factor," Discrimination Factor $= \frac{Sr^{90} \text{ conc. factor}}{Ca^{45} \text{ conc. factor}}$. In absence of discrimination of one isotope relative to that of another the discrimination factor would be unity.

These calculations have been made for 3 species of fresh water fishes (*Danio, Tanicthys,* and *Lebistes*) over a wide range of sodium ion concentrations (Table I). At low concentrations of sodium ion, all 3 species of fresh water fishes discriminate against strontium-90 as compared to that of calcium-45 by factors ranging from 0.921 to 0.964. However, as sodium ion concentration increases, discrimination of strontium-90 becomes less and is essentially absent for all 3 species of fishes at sodium ion concentrations greater than 20 mM/liter. However, in no instance does the discrimination of strontium-90 occur to a greater extent than 0.920 for our experiments.

Similar calculations have been made for *Lebistes* over a wide range of strontium and calcium chlorides (Table II). At low concentrations of alkaline earth elements, *Lebistes* discriminate against strontium-90 relative to that of calcium-45 by factors ranging from 0.937 to 0.949. Discrimination is no longer apparent when water contains more than 3.6 mM calcium/liter or 9.6 mM strontium/liter. The lowest discrimination factor observed (0.937) again demonstrates that fresh water fishes have little ability to distinguish between strontium and calcium.

Discussion. In previous experiments, performed with tap water and individual isotopes (1,2), we obtained discrimination factors for strontium-90 relative to calcium-45 that ranged between 0.93 and 1.03. These results, with factors ranging around 1 are substantiated in the present report and indicate that fresh water fishes similarly metabolize alkaline earth elements. The present experiments, using double tracer technics, eliminate variables such as age, physiological state of animals and variations of water composition which are difficult, if not impossible to control. With double tracer technics, each animal acts as its own control. These experiments, however, demonstrate that fresh water fish may distinguish between strontium and calcium when water contains very low concentrations of salts. Since addition of sodium ion tends to eliminate discrimination in a manner similar to that of strontium or calcium, discrimination appears to be a function of osmoregulatory mechanism. That ionic composition of water plays an important role in uptake of ions by fresh water fishes has recently been

TABLE II. Effect of Calcium and Strontium Chlorides on Rate of Uptake of Ca^{45} and Sr^{90} and Discrimination of Sr^{90} by Male *Lebistes*.

Salt	Conc., mM/l	Rate of uptake ± S.E. Ca^{45} 10^3 cpm/100 mg/day	Rate of uptake ± S.E. Sr^{90} 10^3 cpm/100 mg/day	Discrimination factor ± S.E.	"t"†	P
Basic		91.2 ±27.4	104.1 ±30.7	.937 ± .003 (6)*	17.19	<<.01
Calcium	.45	10.5 ± 1.36	11.7 ± 1.59	.939 ± .016 (8)	5.39	<.01
	3.60	2.24 ± .13	3.52 ± .27	.977 ± .010 (7)	2.29	>.05
	14.5	1.18 ± .18	1.88 ± .29	.974 ± .006 (3)	1.49	"
Strontium	.30	18.9 ± 2.34	24.4 ± 3.32	.949 ± .003 (8)	14.41	<<.01
	9.6	1.53 ± .13	1.92 ± .14	.958 ± .019 (8)	2.14	>.05
	14.6	1.38 ± .17	1.86 ± .19	.968 ± .023 (8)	1.54	"

* Values in parentheses indicate No. of fishes analyzed.

† "t" values calculated from differences of concentration factors for Ca^{45} and Sr^{90}. P values indicate probability of discrimination factor being significantly different from 1.0.

Water activities: $Ca^{45} = 6.19 \times 10^4$ cpm/ml; $Sr^{90} = 1.51 \times 10^5$ cpm/ml.

shown by Phillips *et al.*(11). These investigators found that small amounts of calcium, magnesium or sodium added to a variety of different types of fresh water markedly alters incorporation of cobalt-60 in the gills of trout. It is well known that the gills and integument of fishes play a major role in absorption and excretion of ions, active processes accompanied by corresponding expenditure of energy to maintain osmoregulation in a steady state(12). On the other hand, we previously showed that the spine:body ratio of accumulated strontium-90 in *Lebistes* is not altered by concentration of strontium or calcium in water in which the fish swim, while spine:body ratio of accumulated calcium-45 increases with increasing calcium concentration of water(13). This suggests that bone discriminates against calcium rather than strontium. The divergent results obtained in fish and mammals require further study, and comparisons between animals with such diverse physiology may be hazardous.

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Metabolic Basis of Calcium and Strontium Discrimination: Studies with Surviving Intestinal Segments.* (25738)

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Biological membranes, such as those of intestinal tract, kidney, mammary gland, and placenta, apparently can differentiate qualitatively in the same way between calcium and strontium; calcium always moves more rapidly across the membrane system than does strontium(1-3). Recently, Samachson(4) also observed that Ca^{45} moves preferentially to Sr^{85} across membranes involved in cerebrospinal fluid formation. Little attention has been given to the mechanism involved but previous observations that differential movement occurred in passage from dam to fetus and not from fetus to dam suggested that metabolic processes might be involved(2). In the present study, the differential movement of Ca^{45} and Sr^{85} across intestinal membranes was studied directly, using surviving segments of duodenum and ileum of the rat, for the purpose of determining whether the metabolic integrity of the membrane is essential for discrimination to occur.

Methods. The technic was based upon that of Schachter and Rosen(5), who demonstrated that Ca^{45} can be transported against a concentration gradient by surviving everted duodenal sacs from rats and rabbits. Carworth male rats, weighing about 150-170 g, were fasted overnight prior to experiment. Except for first experiment, all rats were maintained on a low calcium diet for about one week. Rats were killed by exsanguination, the duodenum resected at the pylorus distally, and the ileum from ileo-cecal junction proximally. Intestinal segments (5-6 cm long) were washed, everted and rewashed in cold isotonic saline, tied at one end with suture, injected with 0.6 ml of incubating solution, and the other end tied. The intestinal sacs were placed into 25-ml Erlenmeyer flasks, followed by 3 ml of cold incubating medium. A small plastic vial containing filter paper saturated with 6N NaOH for removal of metabolic CO_2 was suspended in the flask. Flasks were gassed 1 minute with O_2, stoppered, and placed in shaking water bath with temperature controlled at 37°C. Maximum incubation was 2 hours. The incubating medium was that of Schachter and Rosen(5) and consisted of NaCl, 0.135M; KCl, 0.011M; $CaCl_2$, 4 x 10^{-5}M; sodium phosphate buffer at pH 7.4, 0.008M; 0.04 μc Ca^{45}; and 0.02 μc Sr^{85}/ml. At end of incubation, residual volume of solution within the sac was determined. Radiocalcium (Ca^{45}) was counted by direct plating on stainless steel planchets with a thin-windowed Geiger tube (self absorption corrections were unnecessary). Radiostrontium (Sr^{85}) was determined in well-type scintillation detector according to usual procedures. Data are presented primarily as ratio of counts/minute/ml on serosal side (inside of sac) to counts/minute/ml on the mucosal side (outside of sac); this ratio will be abbreviated as S/M after Rasmussen(6). Comparative behavior of alkaline earths is given in terms of ratio of Sr^{85}/Ca^{45} on the serosal side to that of Sr^{85}/Ca^{45} on the mucosal side.

Results. Two different procedures were used to obtain complementary information on behavior of Ca^{45} and Sr^{85} in the *in vitro* system. One procedure was to label mucosal and serosal solutions with equal concentrations of radionuclides; the observations then yielded data only on ability of the membrane to transport either alkaline earth against a concentration gradient as indicated by S/M value other than unity. The second procedure was to label only the mucosal solution and observe the movement of Ca^{45} and Sr^{85} across membrane to the unlabeled serosal side; here, relative rates of movement of the 2 ions across the membrane may be due to several processes such as diffusion or "active" transport. If both procedures were carried to equilibrium, one would expect the same final S/M values;

* This study performed under contract with U. S. Atomic Energy Comm. The counsel of Dr. C. L. Comar is gratefully acknowledged, and technical assistance of Willy Zessoules and A. A. Cross, Jr.

TABLE I. Effect of Dietary Calcium Levels on Movement of Ca^{45} and Sr^{85} in Surviving Duodenal Sac.*

Dietary history	Ca^{45} (S/M)	Sr^{85} (S/M)	Sr/Ca†
Low calcium diet	3.2 ± .5	.9 ± .1	.28 ± .03
High " "	1.7 ± .2	.9 ± .05	.53 ± .02

* Values represent mean ± stand. error of mean; 6 sacs/value; incubation time, 2 hr; Ca^{45} and Sr^{85} in mucosal and serosal solutions; other conditions given in text.

† Sr/Ca = Sr^{85}/Ca^{45} (serosa) ÷ Sr^{85}/Ca^{45} (mucosa).

most of our studies, however, were not carried this long.

In the first study, influence of dietary calcium level on serosal:mucosal ratio (S/M) of Ca^{45} and Sr^{85} in surviving duodenal intestinal sac was determined. Both groups of rats were on their respective diets for 1 week; low calcium diet contained about 0.2% Ca and high calcium diet about 2.0% Ca. Radionuclides were placed both inside and outside of sac. The data (Table I) show 3 important points: (a) that Ca^{45} was transported against a concentration gradient as shown by S/M > 1; (b) that duodenal segments from rats on low calcium diet were capable of maintaining a higher S/M for Ca^{45} than those on high calcium diet; and (c) that Sr^{85} was not transported against a concentration gradient. The difference in behavior of Ca^{45} and Sr^{85} resulted in Sr^{85}/Ca^{45} ratios of 0.28 and 0.53 in segments from low calcium and high calcium diet, respectively.

The time-course of transfer of Ca^{45} and Sr^{85} across duodenal and ileal segments was then studied; radionuclides were placed only in the outside (mucosal) solution. Fig. 1 shows that both radionuclides moved more rapidly across duodenal sac than across the ileal sac. This agrees with *in vivo* measurements since rate of absorption of both Ca^{45} and Sr^{85} from the duodenum exceeds absorption of these radionuclides from the ileum, and radiocalcium always moved faster than Sr^{85}, in agreement with observations in living animal (Lengemann, F. W., private communication).

The effect of incubating duodenal and ileal segments under nitrogen or at 4°C was determined (Table II). Radionuclides were placed within and without the sacs. The duodenal segment at 37°C and under O_2 gave data similar to that of Table I; the ileal segment

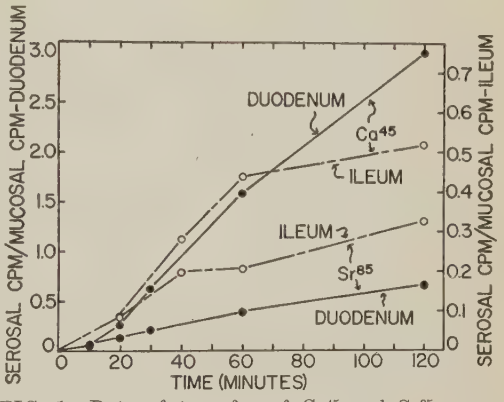

FIG. 1. Rate of transfer of Ca^{45} and Sr^{85} across surviving duodenal and ileal segments; duodenum (—●—), ileum (—○—). Note: S/M ratio for duodenum given on left-hand ordinate; S/M ratio for ileum given on right-hand ordinate.

under same conditions behaved differently. In the ileum, Ca^{45} was not significantly transported against a concentration gradient but an apparent net transfer occurred for Sr^{85} from serosal to mucosal side (S/M = 0.7). This we repeatedly confirmed. Incubation of duodenal and ileal segments under N_2 completely eliminated transport of Ca^{45} in the duodenum (M → S) and eliminated transport of Sr^{85} in the ileum (S → M). At

TABLE II. Effect of Temperature and Gaseous Phase on Metabolism of Ca^{45} and Sr^{85} in Surviving Intestinal Segments.*

Temp. of incubation, °C	Gaseous phase	Duodenum Ca^{45} (S/M)	Duodenum Sr^{85} (S/M)	Duodenum Sr/Ca†	Ileum Ca^{45} (S/M)	Ileum Sr^{85} (S/M)	Ileum Sr/Ca†
37	O_2	3.0 ± .08	1.0 ± .02	.33 ± .02	1.2 ± .01	.7 ± .02	.57 ± .02
37	N_2	1.1 ± .02	1.0 ± .03	.91 ± .03	1.1 ± .01	1.0 ± .02	.91 ± .03
4	O_2	1.0 ± .01	.9 ± .08	.90 ± .03			

* Values represent mean ± stand. error of mean; 6 sacs/value; incubation time, 2 hr; Ca^{45} and Sr^{85} in mucosa and serosa solutions; other conditions as before.

† Sr/Ca = Sr^{85}/Ca^{45} (serosa) ÷ Sr^{85}/Ca^{45} (mucosa).

TABLE III. Influence of Metabolic Inhibitors on Movement of Ca^{45} and Sr^{85} in Surviving Intestinal Segments.*

Treatment‡	Duodenum Ca^{45} (S/M)	Duodenum Sr^{85} (S/M)	Duodenum Sr/Ca†	Ileum Ca^{45} (S/M)	Ileum Sr^{85} (S/M)	Ileum Sr/Ca†
Control	1.9 ± .2	.6 ± .04	.34 ± .03	.8 ± .02	.3 ± .02	.41 ± .05
Sodium cyanide	.7 ± .02	.7 ± .01	1.05 ± .04	.8 ± .04	.8 ± .04	.98 ± .03
Iodoacetic acid	.9 ± .05	.7 ± .02	.71 ± .05	.8 ± .04	.8 ± .03	.99 ± .02
2,4-Dinitrophenol	.7 ± .02	.7 ± .03	1.00 ± .03	.7 ± .01	.7 ± .03	.96 ± .03

* Values represent mean ± stand. error of mean; 6-7 sacs/value; incubation, 2 hr; Ca^{45} and Sr^{85} radioactivity in mucosa solution only; other conditions as before.

† Sr/Ca = Sr^{85}/Ca^{45} (serosa) ÷ Sr^{85}/Ca^{45} (mucosa).

‡ Concentration of inhibitors as follows: NaCN, 5×10^{-4} M; Na iodoacetate, 3×10^{-4} M; 2,4-dinitrophenol, 5×10^{-5} M.

4°C under O_2, there was no transport of Ca^{45} against a concentration gradient. Discrimination between Ca^{45} and Sr^{85} in the duodenum was a result of "active" transport of calcium from M → S, giving a Sr^{85}/Ca^{45} ratio of 0.33. However, discrimination between radionuclides in the ileum resulted from transport of Sr^{85} from S → M with no concomitant transport system for Ca^{45}; Sr^{85}/Ca^{45} ratio for ileum was 0.57.

The influence of sodium cyanide, iodoacetic acid, and 2,4-dinitrophenol on the transfer mechanism and on discrimination in duodenal and ileal sacs also was determined (Table III). Radionuclides were placed only in the outside (mucosal) incubating medium. Since these studies were not run to equilibrium, one would not expect to obtain S/M values for Ca^{45} and Sr^{85} in the duodenum of about 3 and 1, respectively, as before. In duodenal preparations, NaCN, iodoacetic acid, and 2,4-dinitrophenol decreased the rate of Ca^{45} transport but had no observable effect on Sr^{85} movement; in absence of inhibitor, Ca^{45} attained an S/M ratio of about 1.9. Thus, there was elimination of discrimination with NaCN and 2,4-dinitrophenol, and a substantial reduction in discrimination in presence of iodoacetic acid. The effect of inhibitors on the ileal segment was decidedly different; here, inhibitors did not influence Ca^{45} movement but caused a net increase in Sr^{85} movement from mucosa to serosa by blocking the metabolically mediated backflow (S → M) of this radionuclide. Again, the net effect of inhibitor eliminated discrimination between calcium and strontium in the ileum.

Discussion. Extrapolation of *in vitro* studies, like the above, to the intact animal must always be done with caution. However, there are similarities in metabolism of the duodenum and ileum under both situations, as follows: (a) alkaline earth ions move across duodenum more rapidly than across the ileum in both intact animal and *in vitro* situation, (b) Ca^{45} moves across both segments more rapidly than Sr^{85} *in vitro* and *in vivo,* (c) in the ileal segment, Sr^{85} was transferred more rapidly from serosa to mucosa than Ca^{45}, which corresponds to similar observations made by Singer *et al.*(7) in the intact dog. Yet, inconsistencies appear to exist in behavior of alkaline earths in *in vitro* and *in vivo* situations; additional studies may resolve these questions.

Although the term "active" transport is used in the present study, it is realized that existence of such a transport system for calcium has not been conclusively established. That an ion can be transferred against a concentration gradient, and maintenance of concentration gradient by a biological membrane, are not sufficient evidence to postulate existence of transport or carrier system. Regardless of the mechanism, however, the above data demonstrate that calcium-strontium discrimination in surviving intestinal segments is dependent upon a metabolically active membrane.

Summary. (1) The differential movement of Ca^{45} and Sr^{85} across surviving intestinal segments of the rat was studied. (2) In the duodenal segment, Ca^{45} but not Sr^{85} was transported against a concentration gradient from mucosa to serosa. (3) In the ileal segment, Sr^{85} but not Ca^{45} was transferred against a concentration gradient from serosa to mucosa. (4) Incubation of segments at

0°C, under N_2, or in presence of metabolic inhibitors eliminated "active" transport and eliminated strontium-calcium discrimination. (5) Ca-Sr discrimination under given conditions was dependent upon a metabolically active membrane.

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Effect of Guanylic Acid on Plasma NEFA Response to Stress.* (25739)

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A physiological role for the guanine nucleotides in the stress response is suggested by experiments in which pretreatment with these agents significantly extended the swimming time of heavily weighted normal rats(1). We have investigated the effect of guanylic acid on plasma NEFA response to stresses of fasting and tourniquet application in normal and adrenalectomized rats, since this is one metabolic parameter of stress response.

Methods. Male albino rats of the Sprague Dawley strain, weighing 210-230 g were used in most experiments. Male mongrel dogs were used in a few experiments. The rats were maintained on Purina rat chow and water *ad libitum* with the exception of the adrenalectomized animals which were given 0.9% saline in place of tap water. Bilateral adrenalectomy was performed under ether anaesthesia and the animals were not used until at least 7 days postoperative. The fasting period used was 16 hrs. Each experimental and control group contained 8 animals. In the first experiment, normal and adrenalectomized animals were pretreated for 7 days with a daily subc. injection of either 0.3 mg/kg or 1 mg/kg hydrocortisone and then fasted. In the second experiment, normal and adrenalectomized animals were pretreated with only one subc. injection of 1 mg/kg hydrocortisone immediately preceding fasting period. In the third experimental group, normal and adrenalectomized animals were pretreated with 10 mg/kg guanylic acid (racemic sodium guanylate) subc. immediately preceding fasting period. Plasma NEFA levels were determined at end of fasting period. In series 4, tourniquets were applied across both hind limbs just below the inguinal ligament, using a firmly fixed wire clamp. Normal and adrenalectomized animals were used. In these experiments, one group of rats was pretreated with guanylic acid (10 mg/kg) daily, subc., for 7 or 14 days prior to application of tourniquet. A second group received only one injection of the drug on day of experiment. In all cases, the tourniquet was kept in place for 5 hr and then removed. No food was available during this period nor for 10 hr after removal of tourniquet. At the end of this time blood was drawn for analysis and plasma NEFA levels determined. In all experiments blood was taken from the abdominal aorta via a plastic cannula, under ether anaesthesia. NEFA levels were determined by the method of Dole(2). Survival time of the adrenalectomized animals subjected to tourniquet stress was measured after removal of tourniquet. Blood sugar levels were measured in normal dogs at hourly intervals after 10 mg/kg guanylic acid was administered. Simultaneous blood pressure and E.C.G. measurements were made.

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TABLE I. Effect of Hydrocortisone or Guanylic Acid on 16 Hr Fasting Plasma NEFA Levels in 8 Rats/Series.

	meq/l ± S.D.
Intact, untreated	1.113 ± .128
Adrenx, "	1.550 ± .149
Hydrocortisone treated	
1 mg/kg, 1 day	.997 ± .082
" , 7 days	.980 ± .107
Adrenx + .3 mg/kg, 7 days	1.739 ± .137
" + 1.0 " , "	1.006 ± .128
" + 1.0 " , 1 day	.964 ± .085
Guanylic acid treated 10 mg/kg, 1 day	.883 ± .149
Adrenx + 10 mg/kg, 1 day	.794 ± .091

Results. Pretreatment with a physiologic dose of hydrocortisone (1 mg/kg) for either 7 days prior to the 16 hr fast or by single injection immediately preceding the fasting period, restored to normal the NEFA response of the adrenalectomized rats ($P = < .01$, Table I). The smaller dose of hydrocortisone (0.3 mg/kg) was ineffective. In the normal animal, hydrocortisone had an insignificant effect on the characteristic NEFA elevation due to fasting (Table I). Pretreatment of normal and adrenalectomized rats with guanylic acid abolished the usual rise in NEFA levels following fasting ($P = < .01$, Table I). When tourniquets were applied to normal rats, they all survived the stress but plasma NEFA levels remained elevated 10 hours after removal of the tourniquet. Either chronic or acute pretreatment with guanylic acid prevented this rise in plasma NEFA resulting from the tourniquet stress (Table II). All adrenalectomized rats ultimately succumbed to tourniquet shock but 14 days pretreatment with guanylic acid significantly extended survival time of these animals (Fig. 1). Irritability induced by the tourniquet was markedly diminished in those animals which had been treated with guanylic acid.

TABLE II. Effect of Guanylic Acid on NEFA Response of Intact Rats to Tourniquet Stress. 8 rats/series.

Exp. and dose	NEFA, meq/l ± S.D.
Control, untreated	1.119 ± .144
Tourniquet, untreated	1.616 ± .076
" , 10 mg/kg, 7 days	.923 ± .044
" , " , 1 day	1.013 ± .091

In nonfasting dogs treated with guanylic acid, depression of the NEFA levels from 0.689 meq/l to 0.488 was not accompanied by a significant change in blood sugar levels. The drug had no significant effect on blood pressure or E.C.G. of the dog.

Discussion. The mechanism whereby stress elicits an elevation in plasma NEFA levels is not clear. Release of catechol amines as well as cortical steroids may contribute to this response. Glucose utilization, rather than blood glucose levels *per se*, seems to correlate with release of the nonesterified fatty acids. However, hypoglycemia is usually accompanied by an elevation in plasma NEFA(3). In the adrenalectomized animal, medullary catechol amines as well as cortical steroids are absent. Despite this, the elevation in plasma NEFA levels in stressed adrenalectomized animal is greater than in normal controls. The contribution of the sympathetic nervous system to this response has not been clarified. The hypoglycemia of the stressed adrenalectomized animal is probably a major factor. Treatment with hydrocortisone restores to normal the NEFA response to fasting in the adrenalectomized rat but has little or no effect on the intact animal when administered in physiologic amounts. The same amount of hydrocortisone will also maintain adequate blood sugar levels in the adrenalectomized preparation but has little or no effect on blood sugar of normal animals(4). Guanylic acid seems to affect plasma NEFA levels in the normal animal without a concomitant effect on blood glucose levels. Blood pressure and

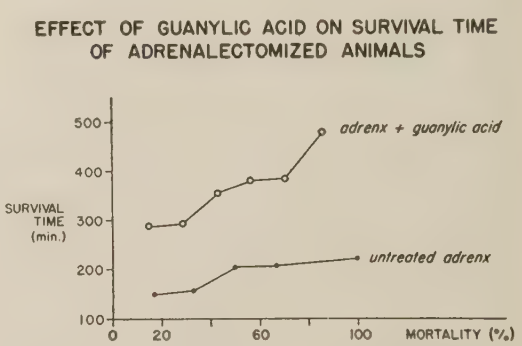

FIG. 1. Chart showing effect of guanylic acid on survival time of adrenalectomized animals subjected to tourniquet stress.

E.C.G. measurements in the dog did not significantly change during the period in which the NEFA levels were falling.

Pretreatment with guanylic acid abolished the NEFA elevation seen in either fasting or tourniquet stress in the intact animal. It was more effective than hydrocortisone in preventing the NEFA rise seen in fasting adrenalectomized animals. Our preliminary data also suggest that pretreatment with guanylic acid significantly extends survival time of traumatized adrenalectomized animals. We are repeating these experiments with addition of guanylic acid injected during the stress period and the period following removal of the tourniquet since availability of this agent was presumably diminishing in the experiments reported.

The mechanism of the effects of guanine derivatives is unknown. The guanine nucleotides are known to participate in formation of the nucleic acids(5). Recent evidence indicates that they may also act as coenzymes in the system that catalyses the phosphorylation of ADP coupled to breakdown of succinyl CoA(6). In addition, guanosine triphosphate has been shown to activate the enzyme systems for fatty acid oxidation, independently of ATP. Of special interest is the finding that lymphatic tissue contains unusually high concentrations of guanine compounds. One of the invariable consequences of acute stress is release of adrenal steroids followed by involution of lymphatic tissues. It is possible that this mechanism releases nucleotides into the circulation and makes them available for protective effects on other organ systems. Once maximal involution of lymphatic tissue has occurred, additional exogenous steroids would presumably be ineffective in this regard. Additional guanine derivatives, however, might continue to contribute to increased resistance to stress. This would explain the results obtained by Kokas, *et al.*(1) in which cortical steroids had no effect on swimming time of weighted intact rats but treatment with guanine compounds increased swimming time 5-fold. We are investigating the response to other stresses under these conditions.

Summary. Pretreatment with hydrocortisone restores plasma NEFA response of adrenalectomized animals to normal but is without effect on intact rats. Pretreatment with guanylic acid, a purine nucleotide, abolishes NEFA response to fasting and tourniquet stress in the normal animal. It prevents elevation in plasma NEFA in the fasting adrenalectomized rats. Survival time of adrenalectomized animals subjected to tourniquet stress is significantly prolonged by pretreatment with guanylic acid. A physiologic mechanism is suggested which operates via lymphatic release of nucleotides resulting from cortical steroid activity.

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Accumulation of Cholesterol in Inositol Deficiency. (25740)

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Inositol, an essential cellular component occurring in almost all types of cells, is an essential nutrient to various organisms such as strains of yeast, fungi, a mutant *Neurospora crassa*, mice, rats, cotton rats, hamster

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and chick. However, virtually nothing is known about the exact role of inositol except that it is an important constituent of lipid (1-3). Gavin and McHenry(4) noted that inositol prevented accumulation of fat and cholesterol in liver of rats, and were confirmed by subsequent workers(5-9). The lipotropic effect of this vitamin in human beings was studied by Abel and co-workers(10-12), who showed that gastrointestinal cancers invariably led to infiltration of fat in the liver. Administration of inositol post-operatively lowered the fat content to normal levels. Though there appears to be general agreement that inositol is a lipotropic factor, there is no agreement as to the mechanism involved and limitation of lipotropic potency. This report deals with the role of inositol particularly in relation to cholesterol metabolism. The γ-isomer of hexachlorocyclohexane (gammexane) is an antimetabolite of inositol. Kirkwood and Phillips(13) showed growth inhibiting effect of γ-isomer and its reversal by inositol. Sarma(14) first observed that gammexane increased cholesterol content of tissues of rice moth larvae (*Corcyra cephalonica* strain). It was of interest to find out whether free or esterified fraction of total cholesterol was increased during gammexane feeding. The effect of gammexane feeding has therefore been studied in both *Corcyra cephalonica* and albino rats.

Material and methods. 1. *Effect of gammexane and inositol on cholesterol content of larvae, Corcyra cephalonica.* Gammexane was obtained from I.C.I., Madras. Experimental procedure was that of Sarma(14) except that gammexane was added at higher levels. Rice moth larvae were fed on 4 types of diets. Diet I was the basal diet, 2 parts wheat + 1 part potato starch. Diet II, basal diet + 4 mg % of gammexane. Diet III, basal diet + 4 mg % of gammexane + 100 mg % of inositol. Diet IV, basal diet + 100 mg % of inositol. Results are presented in Table I.

TABLE I. Effect of Gammexane and Inositol on Cholesterol Content of Rice Moth Larvae *(Corcyra cephalonica)*.

mg/100 g of tissue.

Diet	Wt of 10 larvae on 25th day (mg)	Cholesterol content Free	Ester	Ratio E/F
I	250 ± 2.4	32 ± 1.1	48 ± 1.9	1.5
II	80 ± 1.2	141 ± 1.7	39 ± 1.3	.28
III	200 ± 2.3	52 ± 1.4	68 ± 1.4	1.31
IV	265 ± 3.1	34 ± .2	54 ± 1.3	1.60

Avg of 8 batches.

TABLE II. Effect of Gammexane and Inositol on Cholesterol Content of Serum of Albino Rats.

mg/100 ml serum.

Group	Cholesterol content Free	Ester	Ratio E/F
I	40.5 ± .46	76 ± 4.0	1.9
II	142.3 ± 5.4	58.6 ± 5.1	.41
III	70.0 ± 5.0	135.8 ± 9.9	1.94
IV	46.3 ± 3.3	80.2 ± 5.4	1.73

Avg of 8 values.

2. *Effect of gammexane and inositol on cholesterol content of serum of albino rats.* Rats weighing 60-70 g were divided into 4 groups of 8 rats each. They were housed in individual cages and given the following diets: Group I, casein 12%, groundnut oil 8%, salt mixture A 2%, salt mixture B 2%, Vit. B complex 1%, fat soluble vitamin 2%,‡ sucrose 72.6%, and choline 0.4%; Group II, basal diet + 1 mg "gammexane"/g diet; Group III, basal diet + 1 mg gammexane/g of diet + 4 mg inositol/g diet; Group IV, basal diet + 4 mg inositol/g of diet. Animals were fed for 4 weeks, and all received the same amount of diet. At end of fourth week the animals were killed; blood was taken by heart puncture. Serum was separated by centrifugation and analysed(15) as before for free, total, and ester cholesterol contents. Results are given in Table II.

Results. Rats which received diet containing gammexane (Group II), exhibited hyperirritability, hyperactivity, retardation of growth and mild alopecia. Some animals died and others showed no gain in weight. Addition of inositol seemed to combat slightly the growth depressant effect of γ-isomer. There is a marked increase of cholesterol in both larval tissues and serum of rats. The increase is found to be mainly in free cholesterol and the ester fraction is low, thereby bringing a marked change in ratio between ester (E) and free cholesterol (F). This effect of gammexane is almost completely reversed by inositol in the levels used. Thus inositol appears to

‡ Vitamin mixtures and salt mixture(16).

have greater influence on esterification of cholesterol.

Whether gammexane brings about inhibition of cholesterase, the enzyme system responsible for esterification of cholesterol in liver(17) and pancreas(18), in a manner reversible by excess inositol is under investigation.

Lowering of total cholesterol and increase of ester fraction has been observed by Avigan and Steinberg(19) by feeding rats with unsaturated fatty acids. Klein(20) demonstrated that serum cholesterol is preferentially esterified with poly-unsaturated fatty acids. Therefore the possible role of inositol directly or indirectly influencing the liver to bring about unsaturation of fatty acid deserves further study.

Summary. Tissues of *Corcyra cephalonica* and serum of albino rats fed a diet containing gammexane accumulated more free cholesterol with concomitant decrease in ester fraction. Inositol almost completely counteracted the effect of gammexane. Inositol thus appears to have great influence on esterification of cholesterol.

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Metabolism of Chlorpromazine: Organic-Extractable Fraction From Human Urine.* (25741)

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Despite its widespread clinical use, no detailed data are available on the metabolism of chlorpromazine in humans. Experiments with dogs have led to isolation of chlorpromazine sulfoxide from urine(1) and to renewed emphasis on sulfoxidation as a major metabolic pathway common to the phenothiazines(1,2, 3). More recently, Walkenstein and Seifter (3) have demonstrated that the side chain of promazine (and chlorpromazine) undergoes N-monodemethylation in animals. This finding would probably account for the release of $C^{14}O_2$ following administration of chlorpromazine-(N-methyl)-C^{14} to rats(4).

The present work was undertaken preparatory to a more general study on biochemical and therapeutic significance of variations in

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chlorpromazine metabolism in psychiatric disorders. As indicated below, improved chromatographic methods have enabled the authors to demonstrate 2 new major metabolites in organic extracts of human urine, in addition to other phenothiazine derivatives. The urinary excretion patterns obtained appear to differ significantly from observations based on animal studies in other laboratories.

Experimental. Urine specimens. These were collected from 40 male psychiatric patients at the Creedmoor State Hospital who were receiving 300-600 mg of chlorpromazine orally per day. To eliminate specimens from noncooperative patients, each urine was first screened with Subetest reagent (Subet Laboratories, New York), which was employed to confirm chlorpromazine intake. Control urines were provided by laboratory personnel who were not on medication.

Chromatographic work-up. Urine (100 ml) was mixed with 4 ml of 3 N NaOH and shaken mechanically with ethylene dichloride (120 ml), which proved to be superior to ether as an extractant.† The organic extract was washed with 0.1 N NaOH (60 ml), dried over sodium sulfate and the volume measured before being taken to dryness. The residue was redissolved in 0.003 of its original volume of ethylene dichloride and appropriate aliquots (5 to 50 lambdas) were spotted on Whatman #1 sheets marked "for chromatography." The following 3 chromatographic systems were used: (a) Benzene-acetic acid-water (2:2:1), descending, 21-22°C (temperature critical). For optimal results, the jar (rectangular, provided with paper curtain) was equilibrated over the week-end with both the aqueous phase (below) and organic phase (in trough). A "dry run" was then made with a blank sheet of paper before the spotted sheets were hung from the troughs, using a fresh change of organic phase. After terminating the run, it was possible to re-use the same jar for successive chromatographic separations. (b) Ethylene dichloride-benzene-formic acid (88%)-water (3:1:4:2),‡ descending, 20-22°. The spotted sheets were equilibrated overnight with the aqueous phase. (c) Butanol-ammonium acetate (1 M, pH 7) (2:1), descending. Chromatograms were routinely dipped in Dragendorff-ethyl acetate reagent(5) to visualize the various chlorpromazine metabolites. The following color reagents were also employed: ninhydrin-pyridine dip (ref. 6, p. 66), for detecting primary amines; nitroprusside-acetaldehyde dip(3), for secondary amines; acidic iron dip, prepared from 1 vol. methanol plus 1 vol. $FeCl_3 \cdot 6H_2O$ (1% in 2.5 N HNO_3); and persulfate dip (ref. 6, p. 125). For the spectrophotometric studies, the individual components were eluted from unstained duplicate chromatograms with 0.1 N HCl and read in the Beckman DU spectrophotometer. Methanol was employed for elution when the individual spots were to be rechromatographed. The various standards employed for reference purposes in this study were obtained from the Smith Kline and French Laboratories through the courtesy of Dr. Van Loon.

Results.§ Preliminary studies carried out in our laboratory(7) indicated that organic extracts of urine voided by subjects on CP therapy could be resolved into 3 fractions by paper strip electrophoresis using 1% $KHCO_3$ as the solvent. The first fraction remained at point of application, similar to the behavior of CP. A second fraction showed moderate movement towards the cathode and had a principal UV absorption peak at 255 mμ. The third fraction migrated rapidly to the cathode; in this respect, as well as in its UV

† In our more recent experiments, urine is extracted a second time to remove additional amounts of metabolic material—particularly compound #4, which is voided in large quantities (cf. *Results*).

‡ Separation of organic and aqueous phases is facilitated by permitting the mixture to sit overnight in a separatory funnel. Note that the phases have almost identical specific gravities and may invert with small changes in temperature.

§ The following abbreviations are used: BzA = benzene-acetic acid-water; EdBzF = ethylene dichloride-benzene-formic acid-water; BuAmA = butanol-ammonium acetate; CP = chlorpromazine = 10-(3′-dimethylaminopropyl)-2-chlorophenothiazine; Nor_1CP = desmethylchlorpromazine; Nor_2CP = desdimethylchlorpromazine. Chlorpromazine sulfoxide (CPSO) and its sulfone ($CPSO_2$) are assigned similar prefixes (Nor_1 or Nor_2) to designate desmethyl derivatives.

TABLE I. Comparison between Urinary (Non-polar) Chlorpromazine Metabolites and Several Authentic Chlorpromazine Derivatives.

Substance	R_f (BzA)	Standards with identical R_f's and color reactions*	Color reactions† N	N-a	I	P
Urine						
1	.07		—	—	—	±
2	.16	Nor_2CPSO	++	—	—	+
3	.25(0.24)‡	Nor_1CPSO	±	+	—	+
3′	.25(0.27)‡	CPSO	—	—	—	+
9	.29				—	+
4	.33		—	—	—	+
8	.37		—		—	+
5	.64		—	—	+	+
6	.74	CP	—	—	+	+
7	.87		—	—	?	+
Misc. st'ds						
$CPSO_2$	.40		—	—	—	—
Nor_2CP	.67		++	—	+	+
	.87		—	—	?	+
Nor_1CP	.74		±	+	+	+

* All reference standards were treated like urine, *i.e.*, extracted with ethylene dichloride from aqueous alkaline solution. R_f's obtained in this manner were slightly greater than when standards were spotted directly. Nor_2CPSO, Nor_1CPSO and CPSO were compared with corresponding urine spots in 3 solvent systems, *viz.*, BzA, EdBzF and BuAmA; CP was tested in one system (BzA).

† N = ninhydrin; N-a = nitroprusside-acetaldehyde; I = iron; P = persulfate.

‡ R_f's given in parentheses refer to standards in third column. Color reactions of spots #3 and #3′ were determined after separation with EdBzF.

spectrum, it corresponded to CPSO. However, analytical tests revealed the presence of at least 2 phenothiazine derivatives in the first and third fractions. Further separation via electrophoresis could not be obtained by varying the buffer pH or other parameters. Alternative attempts at a complete fractionation by paper chromatography using butanol-acetic acid-water and BuAmA were also unsuccessful.

A more effective resolution of the urine extracts was obtained upon introduction of the BzA system. Paper chromatography, under the conditions outlined in *Experimental,* yielded 7 discrete spots (#1 to #7, Table I) with both the Dragendorff and persulfate reagents. These substances were shown to be related to chlorpromazine since they did not appear in the normal controls. Furthermore, the first, second and third spots yielded UV absorption maxima similar to CPSO and its desmethyl derivatives, *viz.*, 240, 275, 300 and 340 mμ, in contrast to the spectra obtained from normal urinary chromatograms.

When the third spot was eluted with methanol and rechromatographed with EdBzF or BuAmA, 2 components were noted, designated #3 and #3′. EdBzF proved to be especially useful for separating the sulfoxide metabolites. With this system (as with BzA), CP and related non-sulfoxides were as a rule carried along more rapidly than the sulfoxides. However, the bulk of the Dragendorff-positive material remained behind close to the starting point. By allowing the solvent to run off the paper for a period of 24-48 hours the slow-moving compounds could be effectively separated. This procedure revealed 2 new minor metabolites, #8 and #9. These metabolites flank spot #4 in the BzA chromatogram (Table I) but were not previously detected because of their proximity to this major metabolite. The spots obtained with EdBzF have the following order of increasing R_f's: (#2, #4) < #9 < #3 < #3′ < #8 < (#5, #6).

Ultraviolet studies revealed that 6 compounds, *viz.* #1, #2, #3, #3′, #8 and #9 exhibit the sulfoxide spectrum. Compounds #4 through #7 are apparently not sulfoxides, but their absorption spectra could not be accurately plotted due to background interference from aromatic material normally present in urine. No evidence was found for $CPSO_2$, whose UV absorption maxima (235, 270, 295 and 335 mμ), R_f and persulfate reaction failed to correlate with any of the observed metabolites (Table I).

The ninhydrin and nitroprusside reagents were used to determine whether demethylation had occurred. One compound (#2) reacted as a primary amine and one compound (#3) reacted as a secondary amine. From their R_f's and color reactions the following tentative assignments have been made: #2 = Nor_2CPSO, #3 = Nor_1CPSO, #3′ = CPSO and #6 = CP (cf. Table I). Definitive evidence for presence of Nor_1CP and Nor_2CP on urinary chromatograms could not be found. However, it should be noted that Nor_2CP, as judged by the behavior of the standard supplied to us, was either impure or

unstable in aqueous solution and yielded 2 spots on the chromatogram (Table I). The second, anomalous spot was ninhydrin-negative and apparently an impurity, but it was of interest that its R_f (0.87) and color reactions matched urine compound #7. The significance of these observations and their relevance to origin of compound #7 is now being studied.

Discussion. The findings reported above are not in complete harmony with current concepts concerning the metabolism of chlorpromazine. Although CPSO may serve as an important metabolic intermediate, it constitutes but one (minor) component among the phenothiazine derivatives which may be detected in organic extracts of human urine. Compounds #2, #3 and #4 appear from our studies to be the major extractable products of transformation. Compound #3 (Nor_1-CPSO) was reported earlier in urine of dogs (3). However, the Nor_2sulfoxide (#2) and compound #4 are presented here for the first time. (See *Addendum*)

Though there have been previous indications that monodemethylation (of promazine) occurs in animals to yield Nor_1promazine as well as its sulfoxide, complete demethylation to form the Nor_2 derivatives has been denied (3). In our work with humans we have been unable to demonstrate Nor_1CP, whereas Nor_2CPSO was readily distinguished. The observed discrepancies may be due to difference in species. However, it is noteworthy that Nor_2CPSO is not readily extracted by ether—which is the solvent employed by other workers—and this may account for earlier failures to record this major metabolite.

In addition to the compounds present in organic extracts of alkaline urine, numerous metabolites may be demonstrated in the residual urine(8). These polar derivatives are readily adsorbed on Amberlite CG-50 carboxylic acid resin and are responsible for the purple colors noted when urine is treated with acid containing ferric or nitrite ions. Several compounds present in this second fraction are phenolic derivatives which are linked to glucuronic acid (cf. ref. 9). It therefore appears that the pattern of chlorpromazine metabolism is not quite so simple as previously considered, but involves sulfoxidation, demethylation, ring hydroxylation and other as yet undetermined chemical changes.

During the present study, patients on chlorpromazine therapy tended to differ considerably in their metabolite patterns. Both qualitative and quantitative variations were observed. In an unusual case, urine extracts which gave characteristic sulfoxide UV absorption spectra were later shown to be almost devoid of CPSO and its demethylated products (spots #2, #3 and #3′), in contrast to the relatively abundant substances #1 and #4. Whether this variation is correlated with drug dosage, diet, clinical response to the drug, or psychiatric disorder, or reflects individual differences, has not been determined. Further studies along these lines are indicated.

Summary. Chromatographic studies reveal the presence of 10 Dragendorff (+) compounds in organic (ethylene dichloride) extracts of alkalinized human urine following administration of chlorpromazine. Six of these compounds appear to be sulfoxides. Included in this group are chlorpromazine sulfoxide—which is a relatively minor product—as well as 2 major metabolites whose chromatographic behavior is indistinguishable from Nor_1 and Nor_2chlorpromazine sulfoxide. A third major metabolite (compound #4) remains unidentified, but it is not a sulfoxide derivative. It is indicated that the residual urine remaining after extraction contains numerous additional (polar) metabolites.

We are indebted to Drs. Walter Brinitzer and Raphael Greenberg (Creedmoor State Hosp., Queens Village, N. Y.) for providing urine specimens used herein, to Daniel White and Michael Miller for their assistance during initial phase of this study, and to Drs. E. J. Van Loon and T. H. Lin (Smith Kline and French Laboratories) for their continued interest in our work. TheEdBzf system was kindly suggested by Irving Eisdorfer.

Addendum. Present studies suggest that compond #4 may not be a phenothiazine derivative. This conclusion is based on two observations: (1) The compound has only been observed in a small proportion of patients at the Hillside Hospital, in contrast to the Creedmoor Hospital group; (2) Following its elution from the BzA chromatogram, purification, and

rechromatography in the EdBzf system, #4 appears to lose its persulfate-staining properties. This observed change in staining behaviour has not been noted for the other compounds tested (#2 through #6). There is tentative evidence that the presence of compound #4 in urine may be related to the use of tobacco.

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Effects of Tetrazotized Diorthoanisidine on Depressor Response to 5-Hydroxytryptamine in the Rabbit. (25742)

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Diazo and tetrazo salts react *in vitro* with 5-hydroxytryptamine (5-HT) to form blue colored azo dyes(1). If similar reactions can occur *in vivo* these salts should block the pharmacological actions of 5-HT unless the coupled reaction by-product retains 5-HT activity. Results of previous studies indicate that the tetrazo salt, tetrazotized diorthoanisidine (TDA) is an adrenergic blocking drug (2). The blue colored complex formed by reacting TDA with 5-HT does not possess 5-HT activity on blood pressure of dogs or rabbits even in doses equivalent to 200 μg of 5-HT. We, therefore, decided to determine whether TDA could block some of the effects of exogenously administered 5-HT. Rabbits were used in all experiments since 5-HT produces a hypotensive response in this species. Other workers have shown that sympatholytic agents can modify the pressor effects of 5-HT but do not significantly alter its depressor action in the rabbit(3).

Methods. Albino rabbits weighing 2-3 kg were used. The technic of Horita(4) utilizing the pyretogenic effects of 5-hydroxytryptophane in animals pretreated with iproniazid was used as an index of central effects of 5-HT. Rectal temperatures were recorded at various time intervals in unanesthetized control animals and in others pretreated with TDA. Blood pressure responses to 5-HT, l-epinephrine and l-norepinephrine were recorded continuously from a carotid artery of atropinized, vagotomized rabbits before and after treatment with TDA. These animals were anesthetized with pentobarbital (25-30 mg/kg). Supplementary doses were given as needed.

Results. Pyretogenic action of 5-HT. Iproniazid was administered subcutaneously in 50 or 100 mg/kg doses. Two to 3 hours later 20 mg/kg of 5-hydroxytryptophane were injected intravenously. Rectal temperatures of rabbits so treated were found to be increased markedly within one hour. According to Horita and Gogerty(4) this response is the resultant action of 5-HT accumulation in brain tissue brought about by monoamine oxidase inhibition with iproniazid. A total of 14 rabbits received iproniazid and 5-hydroxytryptophane treatment. Seven of them also were injected intravenously with 20 mg per kg doses of TDA immediately before or following 5-hydroxytryptophane administration. No apparent differences in rectal temperature

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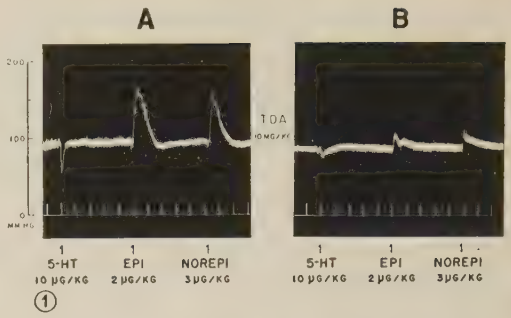
curves, either in rate of ascent or absolute rise, between the 2 groups were noted. Maximum temperature levels were reached 2-3 hr following 5-hydroxytryptophane administration. Mean levels were 41.2 for animals which did not receive TDA and 41.1 for those treated with TDA. Control values for the 2 groups were 39.1 and 38.8, respectively. All but one of the animals died at or near the peak of temperature rise. Arterial blood samples from animals treated with TDA and 5-hydroxytryptophane were blue-colored probably because of coupling between circulating TDA and 5-hydroxytryptophane with formation of an azo dye. In 4 additional experiments TDA (20 mg/kg) alone had no significant effect on rectal temperatures measured up to 4 hours after administration.

Depressor responses to 5-HT. Effects of TDA on depressor response to intravenous administration of 5-HT were studied in 18 rabbits. Response was partially or completely blocked by 10 mg/kg doses of TDA in 14 animals. Larger doses (20-40 mg/kg) were required to produce significant blockade in the remaining 4 animals. A typical experiment is illustrated in Fig. 1.

Panel A of Fig. 1 records the depressor effect of a 10 μg/kg dose of 5-HT. Pressor responses to a 2 μg/kg dose of epinephrine and a 3 μg/kg dose of norepinephrine are also shown. Panel B illustrates alterations in response induced by a 10 mg/kg dose of TDA given intravenously. Only a slight depressor response was obtained on administration of 5-HT 30 minutes following TDA injection. At this time carotid blood pressure level was only slightly lower than that prior to TDA administration. Pressor responses to epinephrine and norepinephrine could still be obtained (Panel B) but were diminished in magnitude. In most experiments TDA produced a sustained lowering of mean blood pressure ranging from 5-30 mm of Hg. The blocking action usually persisted for duration of an experiment (4-6 hr).

The blockade produced by TDA appeared to be competitive and could be overcome by increasing dose of 5-HT. One of 3 equivalent responses to 20 μg/kg injections of 5-HT is shown in panel A of Fig. 2. Accentuation of



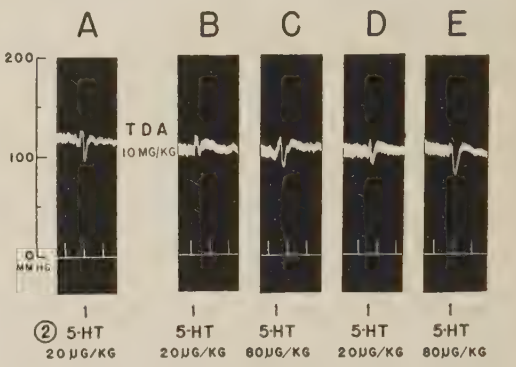


FIG. 1. Blockade of depressor effect of 5-hydroxytryptamine and pressor effects of l-epinephrine and l-norepinephrine by TDA. Time base in min. 35 min. period between A and B.

FIG. 2. Competitive blockade of depressor effects of 5-hydroxytryptamine by TDA. Time base in min. Periods between responses A-B, 1 hr; B-C, 10 min.; C-D, 2 hr; D-E, 10 min.

the initial pressor component and elimination of the depressor effect of 5-HT one hour after TDA administration are shown in panel B. A 4-fold increase in dose 10 minutes later restored depressor component (panel C). Partial antagonism was still evident on administration of the initial 20 μg/kg dose of 5-HT 2 hours after TDA was given (panel D). Readministration of the large dose of 5-HT 10 minutes later again overcame the blockade produced by TDA.

Effects of phenoxybenzamine. Phenoxybenzamine in doses which completely abolished or reversed pressor response to l-epinephrine in 4 rabbits did not significantly affect depressor response to 5-HT.

Discussion. Our data show clearly that TDA can reduce or abolish depressor response to exogenous 5-HT. The receptors involved are not ordinary adrenergic sites since they are not inactivated by phenoxybenzamine and

it can be shown that TDA potentiates depressor response to isoproterenol(2).

The experiments described herein were undertaken to examine the possibility of chemical inactivation of 5-HT by reaction with TDA. Inactivation has been demonstrated but evidence regarding mechanism is merely circumstantial. TDA does react with 5-HT *in vitro* and the reaction product in large doses has no measurable effect on blood pressure. It also blocks the effects of 5-HT on blood pressure in the rabbit but this may be a consequence of binding to receptor sites and not chemical combination with 5-HT. The presence of a blue color in arterial and venous plasma samples of animals treated with 5-hydroxytryptophane and TDA indicates that *in vivo* coupling can occur but does not exclude the possibility of concurrent binding to receptor sites.

The pyretogenic response to iproniazid and 5-hydroxytryptophane combinations was not modified by TDA possibly because TDA does not penetrate into central nervous system tissue in sufficient quantity, or because amount of 5-HT produced was sufficient to override a TDA blockade.

Summary. The tetrazo compound, tetrazotized diorthoanisidine (TDA) reduced or abolished depressor response to 5-HT in the rabbit but did not alter the pyretogenic effects of combined treatment with iproniazid and 5-hydroxytryptophane. The mechanism by which TDA modified blood pressure response to 5-HT is discussed.

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Virus Nature of Infectious Pancreatic Necrosis in Trout. (25743)

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Virus-caused hyperplastic diseases of fishes have long been recognized, and Zhdanov(1) included them with the pox group. They have been omitted from other classifications, and Smith(2) stated that "evidence for the existence of viruses affecting fish does not rest upon so secure a scientific foundation." Except for subsequent work on an infectious disease of a Pacific salmon(3), recognized virus diseases of fishes have been discussed in recent reviews(4,5). Infectious pancreatic necrosis (IPN) of trouts is an acute, virulent, and high-mortality disease occurring typically in very young eastern brook trout (*Salvelinus fontinalis*)(6,7,8). The pancreatic lesions are similar to those produced by Coxsackie viruses in mice(7), and like mice, older fish resist infection(8). On the basis of infectiousness of IPN, histological findings, and absence of other pathogens, a virus has been suggested as causative agent(6). This communication describes transmission, TC propagation, and some characteristics of an agent present in fish with IPN.

Materials and methods. 1. *Fish tissue* was cultivated according to methods of Wolf and Dunbar(9). Cultures were used after explants had developed extensive epithelial sheets, 3 to 4 days. 2. *Mammalian tissue and cell cultures.* HeLa cells (Gey) were cultivated in 80% SM 199 and 20% horse serum with antibiotics. Other tissue or cell cultures were obtained from commercial source,* and furnished with medium specified by supplier. 3. *TC inocula.* Moribund fish symptomatically diagnosed as having IPN were used fresh, after freezing and storage at −20°C, and after preservation in 50% glycerol at about 4°C. They were ground in sev-

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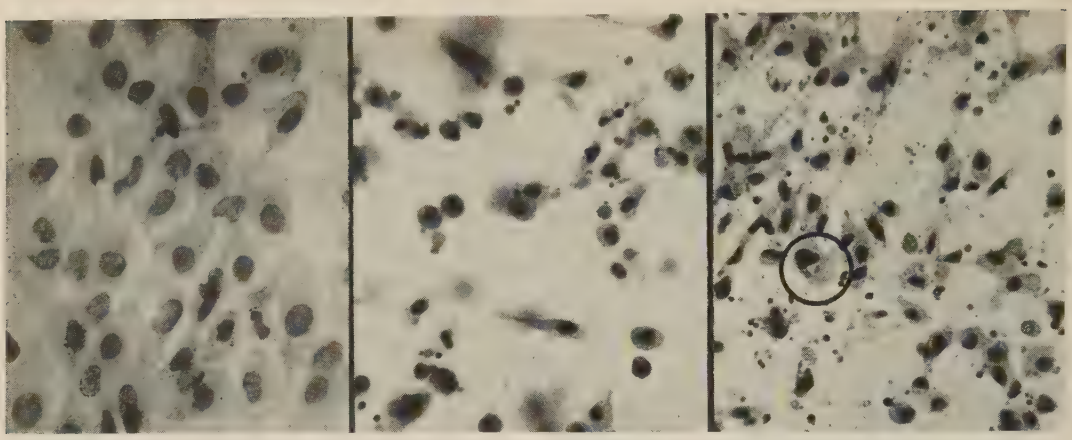

FIG. 1. (Left) Control culture showing normal epithelial sheet from explant of eastern brook trout caudal fin. (Center) Inoculated culture showing degeneration which followed introduction of virus of infectious pancreatic necrosis. (Right) Inoculated culture with advanced necrosis of the cell sheet. Circled area contains cell in which there is an object that is similar to cytoplasmic inclusion bodies seen in histological preparations of pancreas from infected fish. ×280.

eral volumes of SM 199 or Earle's BSS, centrifuged at 12,000 or 26,000 G for 10 to 15 minutes at about 4°C, then filtered through asbestos (Seitz) or porcelain (Coors P3). Inoculum was .01 ml or, more commonly, .1 ml/culture. 4. Two *infectivity trials* were conducted with susceptible fish at 12.5°C, and survivors of each given follow-up feedings of virulent material. A single lot of eastern brook trout was used at 4 weeks and 8 weeks of age. For first trial duplicate jars of 100 4-week-old fish were fed TC harvests of 2 "strains" of IPN in 8th transfer representing 2.6×10^{-10} dilution of primary inocula. Control fish received no IPN, but were otherwise treated identically. Observation was carried out for 30 days when mortality ceased. One hundred and fifty fish which survived exposure to infection were distributed to 2 jars, and 2 other jars each received 90 control fish. All were fed ground victims of IPN. At start of second trial fish were 8 weeks old and considerably larger. They were distributed among 12 troughs, 53 g, average 190 fish/trough. The 12 troughs were randomly subdivided into 4 groups of 3 troughs each. Fish in first group were fed with TC harvest IPN in 6th transfer (1.1×10^{-8} dilution of primary inoculum). Fish in second group were fed pooled ground IPN victims, and fish in third group received a Seitz-filtrate of same pool of ground material. A group of 3 control troughs received no IPN. Five weeks after infection, mortality had almost stopped and observations terminated. Eighty survivors which had been fed IPN victims were distributed equally to 4 jars. Two of these jars were held for possibly recurrent mortality. The 2 remaining jars and 2 jars each with 20 formerly uninfected fish were given follow-up feedings of ground IPN victims. Mortality from all phases was sampled and examined by histological section and/or tissue culture inoculation.

Results. 1. *Propagation and cytopathic effect (CPE) of IPN.* Epithelium from gill, swim bladder, spleen, kidney, and caudal fin of eastern brook trout was consistently destroyed by filtered inocula prepared from fish with IPN. Caudal fin was used for most work, because it was easiest to prepare in uniform explants free of debris. CPE was evident in 18 to 24 hours after primary inoculation, and complete destruction followed in 1 to 4 days (Fig. 1). Fibroblast-like cells were more resistant but underwent some granulation and darkening. CPE usually began as darkening of peripheral cells but was also common as foci within sheets. Necrotic areas enlarged and met, and sheets were usually obliterated. Objects similar to inclusion bodies were found (Fig. 1), but definitive work has not been done. Inocula were prepared from 8 outbreaks of this disease, and all produced characteristic CPE. Log dilutions of primary inocula always produced CPE at 1 ×

10^{-4}, but at 1×10^{-6}, some inocula failed to evoke CPE. Material from 2 sources was passaged 8 and 9 times during period of waiting for seasonally available host fish. Final dilutions were 3.6×10^{-10} and 2.6×10^{-11}, but latter passages with one "strain" were accompanied by reduced CPE, and it was difficult to distinguish inoculated from uninoculated cultures. Inocula were customarily left in cultures throughout incubation. If cultures were merely exposed to inocula for 15 minutes, then washed repeatedly, CPE still occurred. 2. *Characteristics of agent of IPN.* The agent was inactivated at 60°C for 1 hour. Agent stored in victim fish for 4½ years at –20°C and 2½ years in 50% glycerol at 4°C retained its infectivity. It consistently produced CPE in rainbow trout (*Salmo gairdneri*) caudal fin epithelium. In 2 attempts with brown trout (*Salmo trutta*) and 1 with bluegill (*Lepomis macrochirus*) caudal fin epithelium, CPE failed to occur. HeLa cells inoculated with this agent and incubated at 19°, 25° and 37°C, were resistant. Similarly at both 19° and 37°C, agent did not produce CPE in following tissue and cell cultures: human embryonic intestine (Henle), human adult liver (Chang), MAF—human embryo skin and muscle (Gray), KB—human carcinoma nasopharynx (Eagle), Sarcoma 180—mouse sarcoma (Foley), human heart adult (Girardi), monkey heart (Salk), hamster kidney and monkey kidney. 3. *Infection experiment with 4-week-old fish.* Loss in inoculated and control portions during first 5 days of experiment was about equal. Cause was not determined but was not unusual for such young fish. Starting at 7 days, usual incubation period, inoculated fish exhibited whirling and other abnormal behavior characteristic of IPN. Mortality increased in infected portion, but mortality in controls dropped (Fig. 2). Dead fish from inoculated jars showed symptoms of the disease, and IPN was confirmed by histological section. A filtered inoculum prepared from one such dead fish produced typical and rapid CPE in susceptible tissue culture. An additional feeding of infected food was followed by a second, but slight, peak in mortality. At termination, 23% of inoculated fish and 8.5% of control

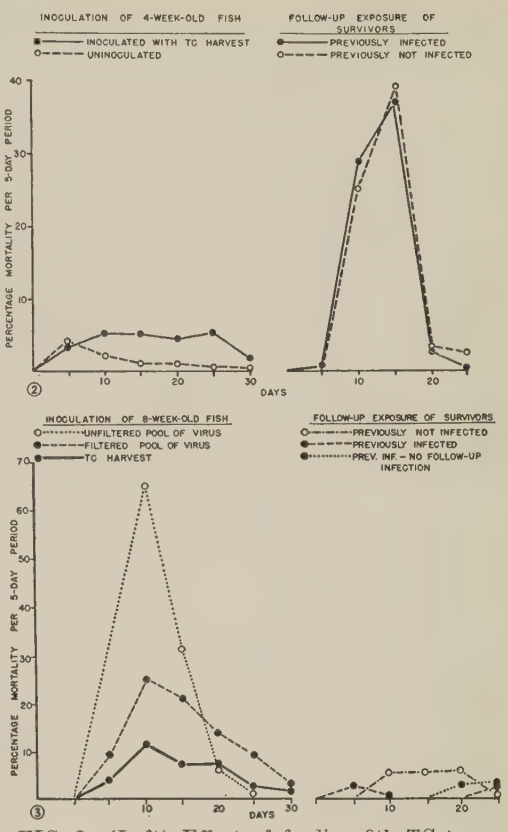

FIG. 2. (Left) Effect of feeding 8th TC transfer of virus of infectious pancreatic necrosis to 4-wk-old eastern brook trout, and (Right) effect of feeding freshly ground victims of an epizootic to the survivors.

FIG. 3. (Left) Effect of feeding 6th TC transfer of virus of infectious pancreatic necrosis, ground victims of the disease, and a bacteria-free filtrate of ground victims to 8-wk-old eastern brook trout. Mortality among uninoculated controls was less than 2%. Its cause was not determined, and curve was not plotted. (Right) Response of some surviving fish to a follow-up feeding of ground victims from 2 epizootics.

fish died. IPN was not found among controls. When given follow-up feeding of infected fish, 57% of controls and 56.5% of previously infected fish died. 4. *Infection experiment with 8-week-old fish.* Initial loss from unknown causes did not occur. Typical IPN behavior was seen in all troughs of infected fish after usual incubation period, and followed by a rapid rise in mortality rate (Fig. 3). At termination, 86% of fish fed unfiltered material died, and loss was 60% among fish fed from filtered portion of this pool. Mortality among TC harvest-fed fish

totalled 30%. IPN was confirmed in all inoculated lots by histological section but could not be found in control fish. Fish were then 13 weeks old. Twenty-five days after the follow-up feedings of infective material 6 of 40 previously uninfected fish had died. Two of 40 formerly infected fish died after the follow-up feedings, but an equal number died in the jars which were not reinfected (Fig. 3).

Discussion. The agent of IPN demonstrated a high degree of specificity and produced CPE only in trout tissue. In contrast, Sanders and Soret propagated eastern equine encephalomyelitis in embryonic fish(10). When primary inocula were diluted CPE was variable or even absent at 10^{-6}. IPN was passaged in TC with concomitant CPE at dilutions of 10^{-11} and 10^{-10}, therefore, *in vitro* propagation of agent must have occurred. Such TC harvests were used to infect fish, but were associated with a low rate of mortality. Lowest mortality resulted from material which had been passaged most. When 2 portions of a virus pool were used to infect fish, the filtrate produced less mortality than the unfiltered portion. Presumably, filtration reduced number of infective particles. If low mortality from TC harvests was due to a low population of infective virus, TC passages could have been mere dilution of original virus. A more likely explanation was that TC passage resulted in virus propagation, but that it was accompanied by alteration. Two facts supported this latter hypothesis. First, through repeated transfer CPE diminished somewhat but could not be a toxicity phenomenon at dilutions used and with specificity shown. Secondly, reisolation of agent from fish infected with TC harvest was accompanied by usual severe CPE. It seemed more likely, therefore, that alteration of virus occurred in TC. Few 13-week-old fish died following infection, confirming reported resistance of older fish(8).

Summary. A filterable, heat labile agent has been found in salmonid fishes with presumptively diagnosed and histologically confirmed infectious pancreatic necrosis. In presence of penicillin, streptomycin, and mycostatin, agent produced CPE in cultures of tissues from susceptible host fishes, but not in cultures from a non-host fish or mammals. Isolates of agent were passaged 8 and 9 times, and last dilutions significantly exceeded extinction point noted in original inocula. Sixth and 8th TC passage material was infective for susceptible trout and produced typical disease syndrome. Inocula prepared from such victim fish also evoked characteristic CPE in fish tissue culture. Agent has thus far survived 4½ years storage at –20°C, and 2½ years in 50% glycerol at 4°C. These facts support the proposition that IPN is a virus-caused disease.

The authors are indebted to Dr. R. E. Lennon, S. W. Watson, T. J. Parisot, and R. G. Piper for reviewing the manuscript.

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Paroxysmal Pulmonary Edema Induced by Intravenous Chloroform.* (25744)

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During other investigations(1), a new method was developed for the production of paroxysmal pulmonary edema in rabbits. This method, consisting of intravenous injection of chloroform, is also effective in dogs. Chloroform has direct depressant effect on the myocardium. This basic observation of MacWilliam(2) was confirmed and extended by others, and extensively reinvestigated by Orth *et al.*(3). The extensive literature on chloroform is summarized in 3 reviews(4,5,6), which deal with history, physiology, and pharmacology of this drug.

Method. Thirty male albino rabbits weighing about 2 kg each and 11 male mongrel dogs weighing 14 and 18 kg were used. The method was based on slow intravenous administration of chloroform in intact unanesthetized animals, properly restrained. In the rabbit, the marginal ear vein was used; in dog the saphenous vein. Preliminary investigations, concerned with speed of administration and effective dosage, demonstrated that best results would be obtained by slow (60-120 min) intravenous injection of 0.1 cc/kg using a graduated Luer-Lock syringe. Larger doses or faster injections cause rapid death in most animals without formation of edema. Smaller doses gave variable results. In some experiments, saline solution (approximately 100 cc/kg) was injected by rapid (less than 5 min) or slow (more than 30 min) infusion, to overload the circulation. This was done either before, during, or after injection of chloroform. This technic, however, did not result in more severe pulmonary edema than chloroform alone. ECG was recorded throughout each experiment using bipolar limb leads, augmented "unipolar" limb leads, and 2 precordial leads (V_4R and V_4). Standardization was 1 MV = 1.5 cm (Fig. 1 and 2). Animals which did not die spontaneously were sacrificed 60 minutes after injection of chloroform, to examine the lungs. Evaluation of pulmonary edema was based on determination of lung-body (L/B) index (weight of lung in g divided by total body weight in g times 100). Average normal value of L/B index is approximately 0.50 in the rabbit and between 0.7 and 0.8 in the dog. An index higher than 1 in either species is evidence of pulmonary edema(1).

Results. Results are summarized in Table I. Average survival time was very close in the 2 species, *i.e.*, about one-half hour. However, 6 out of 11 dogs survived one hour, while only 7 out of 25 rabbits survived one hour.

Immediate response to injection of chloroform was characterized by short period of excitement and struggle, followed by transitory vagal effect on heart and respiration, *i.e.*, bradycardia and bradypnea. No evident anesthetic effect was observed with this dose, but animals appeared depressed and, with few exceptions, remained still. Then, respiration became shallow and labored, and tachycardia occurred. Representative electrocardiograms are presented in Fig. 1 and 2. Terminal pulmonary edema was characterized by appearance of bloody foam from mouth and nostrils, followed by a few gasping respirations, one or 2 generalized convulsions, and death.

At post mortem, essential findings were in lungs, which appeared markedly congested, with various amounts of foam in trachea and bronchi. Average lung-body index was 1.06 for rabbits and 1.33 for dogs. In 25 rabbits, liver-body index was also determined. Normally this index varies between 3.5 and 4.5 as

TABLE I. Results of Chloroform.

Species	No.	Avg survival time, min.	No. surviving at 60 min.	Avg lung/body index	Avg liver/body index
Rabbits	25	32	7	1.06	4.69
Dogs	11	37.5	5	1.33	

* This study aided by Grant of Nat. Heart Inst., U.S.P.H.S.

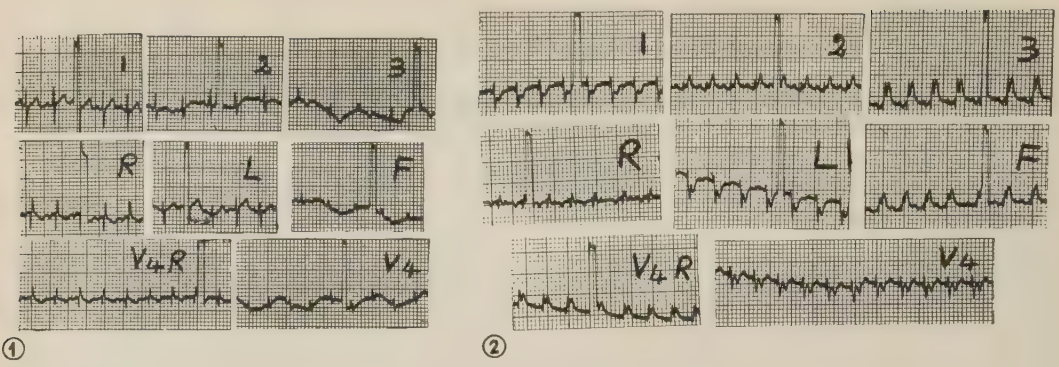

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FIG. 1. Normal electrocardiogram in a rabbit. The extremity leads (1, 2, 3, R, L, F) and 2 precordial leads (V_4R and V_4) are recorded. Standardization is 1 MV = 1.5 cm.

FIG. 2. Same rabbit as in Fig. 1. Electrocardiogram taken 19 min. after intrav. inj. of 0.1 cc/kg of chloroform, showing a pattern of "acute cor pulmonale."

ascertained by previous determinations. An index higher than 4.5 is interpreted as evidence of congestion of liver, and this occurred in all animals. The heart stopped in diastole but appeared normal, except for deep cyanotic hue. Large clots were present in cardiac chambers. Serial sections in 2 rabbits failed to reveal microscopic alterations. Other organs appeared normal.

Intracardiac pressure tracings in dogs revealed gradual decrease of systolic pressures either in both ventricles or only in the left. Later, end-diastolic pressure of both ventricles and mean pressure of left atrium rose. In final stage of most experiments, left ventricular systolic pressure further decreased while that of the right either remained at the same level or rose slightly. Left atrial pressure showed a gradual rise, attaining levels of from 20 to 26 mm Hg (Table II).

The authors believe that pulmonary edema was caused by depressing action of chloroform on myocardium, as demonstrated by Orth *et al.*(6) both in isolated hearts and in intact animals. The results indicate that, by using slow intravenous injections, the primary vagal effect of the drug is minimized and its direct depressing effect on the heart is increased. In final stage, left ventricular depression is greater than the right, pressure in pulmonary capillaries becomes greater than the colloid osmotic pressure of the blood and transudation into the alveoli results.

TABLE II. Intracardiac Pressures (mm Hg) Following I.V. Chloroform in a Dog.

Min. after inj.	0	15	30	45
LV	90/5	70/7	60/8	50/10
LA (mean)	8	10	12	22
RV	20/4.5	22/8	18/8	30/12

Summary. 1) A method for production of acute pulmonary edema in unanesthetized rabbits and dogs, consisting of slow (60-120 min) intravenous injection of chloroform (0.1 cc/kg) is presented. 2) Pulmonary edema, as determined by lung-body (L/B) index, occurred in all animals and was accompanied by congestion of liver. 3) Measurements of intracardiac pressures suggest that pulmonary edema is due to relatively greater drop in left than in right ventricular pressure, causing increased pressure in capillaries of lung. 4) Results are believed due to direct depressant effect of chloroform on the myocardium.

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Intravascular Effect of Heparin on Plasma Nonesterified Fatty Acid and Triglyceride During Alimentary Lipemia. (25745)

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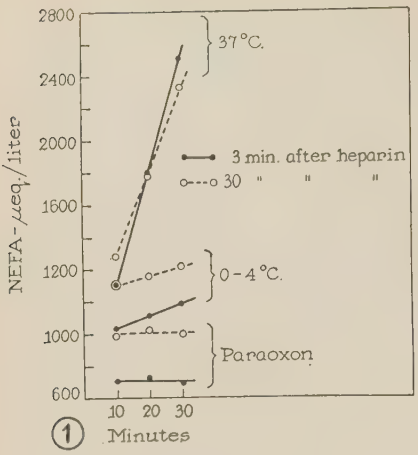
Heparin injected intravenously causes release of lipoprotein lipase into the circulation (1,2), and a decrease in turbidity of lipemic plasma associated with hydrolysis of plasma triglyceride(3). Since this process releases fatty acids(4,5), the level of nonesterified fatty acids (NEFA) has been utilized as index of amount of lipase released by heparin(6,7, 8). It is important to observe that lipolysis, initiated intravascularly, continues in the test tube(6). Lipolysis must be completely abolished after blood is drawn, if intravascular effect of heparin is to be determined. Using a potent lipoprotein lipase inhibitor, p-nitrodiethyl - phenyl - phosphate (paraoxon)*(9), intravascular effect of heparin on plasma turbidity is considerably less than previously observed(10). This inhibitor was utilized in the present study to define changes in plasma NEFA and triglyceride occurring intravascularly in association with the clearing reaction.

Method. Six medical students served as subjects to test methods of eliminating lipase activity from post-heparin plasma. They ingested 240 ml of heavy cream at about 8:00 a. m. Water was allowed *ad lib.* until measurements began 5 hours later. Samples of blood were obtained 3 and 30 minutes after injection of heparin (10 mg intravenously). All samples were collected in tubes containing sodium oxalate as anticoagulant. Each sample was divided into 3 portions: The first portion of plasma was incubated at 37°C after separation in centrifuge at room temperature. The second portion was handled as the first except that paraoxon was added to collecting tubes in sufficient quantity to establish final concentration of 2×10^{-3} molar. The third portion of plasma was separated and maintained at 0-4°C. Aliquots of each of the 3 portions were taken at 10-minute intervals over a 30-minute period and extracted for determination of NEFA. Intravascular effect of heparin was tested in a separate study. Samples of blood were obtained from 12 medical students at 30, 25, 15 and 1 minute before intravenous injection of heparin (10 mg). Additional samples were taken at 5, 15 and 30 minutes after injection. Each sample was divided into 2 aliquots: One was mixed with paraoxon and sodium oxalate, and was processed at room temperature; the other, containing sodium oxalate alone, was kept at 0-4°C. All samples were analysed for NEFA and triglyceride. NEFA was determined by the method of Dole(11), and triglyceride by the method of Van Handel and Zilversmit (12).

Results. Inhibition of lipolysis in vitro. Fig. 1 shows complete inhibition of fatty acid release in post-heparin plasma treated with paraoxon. Cooling caused marked but incomplete reduction in rate of hydrolysis when blood was drawn after injection of heparin, cooled in ice water and separated in refrigerated centrifuge (the whole procedure requiring about 10 minutes). Concentration of NEFA was consistently higher in cooled blood than in aliquot of blood treated immediately with paraoxon and separated at room temperature. The difference between tubes apparently was due entirely to lipolysis in the cooled sample. Paraoxon itself had no effect on determination of pre-existing NEFA; samples of blood, taken before injection of heparin and cooled or treated with paraoxon, showed no significant differences in concentration of NEFA. In contrast to these results, no difference was found in concentrations of triglyceride when samples containing paraoxon were compared with cooled aliquots.

Intravascular effect of heparin. Following injection of heparin concentration of NEFA in plasma (treated with paraoxon) rose 327 $\pm$ 219 μeq/l (Fig. 2). This value is significantly less than the 618 $\pm$ 419 μeq/l rise observed in cooled specimens ($P<0.001$). Con-

* Obtained through courtesy of Dr. Schrader, Farbenfabriken Bayer, A. G., Wuppertal-Elberfeld, West Germany.



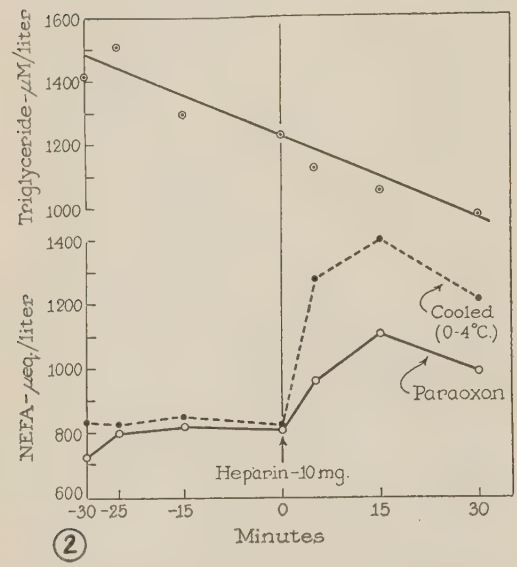


FIG. 1. NEFA concentration of plasma incubated *in vitro*.

FIG. 2. Effect of heparin on NEFA and triglyceride concentration of plasma.

centration of triglyceride in plasma fell throughout period of observation (Fig. 2). Mean rate of decrease before heparin was 9.3 ± 3.2 μM/l min and afterwards was 6.3 ± 2.5 μM/l min. The difference in rates was not statistically significant (P>50).

Discussion. Amount of NEFA released *in vitro* when post-heparin blood was cooled, but is not otherwise treated, corresponded to hydrolysis of less than 100 μM/l of triglyceride, a quantity at borderline of sensitivity of the analytical method. This is sufficient to account for lack of significant difference in triglyceride concentration between cooled and paraoxon-containing specimens.

It is also theoretically possible that hydrolysis of triglyceride might be underestimated. Lipoprotein lipase acts preferentially on the alpha position of triglyceride to form diglycerides(13), and the analytical method used to determine triglycerides does not distinguish between diglyceride and triglyceride. However, post-heparin plasma has been analysed for lower glycerides by silicic acid chromatography(14) and contained less than 2% of total glycerides as diglyceride or monoglyceride even though no precaution was taken to inhibit lipolysis. It seems likely, therefore, that lower glycerides did not constitute a significant fraction of plasma glycerides in the present study.

From data obtained by incubating post-heparin plasma *in vitro*, rate of release of NEFA after injection of heparin was calculated to be 72.8 ± 18.3 μeq/l 3 min after injection and 53.3 ± 12.1 μeq/l 30 min after; these rates are equivalent to complete hydrolyses of triglyceride at rates of 14.6 and 26.1 μM/l min. Since triglyceride appears to be removed from circulation at a rate of 125 μM/l min(15), heparin, at dosage used, would not be expected to have a significant effect on triglyceride concentration of plasma.

The above findings do not support the opinion that heparin significantly accelerates clearance of circulating triglyceride. Earlier studies, seeming to indicate marked reduction in circulating triglyceride after heparin, appear to have been complicated by incomplete inhibition of lipoprotein lipase.

Summary. Clearance of triglyceride from plasma during alimentary lipemia was not significantly influenced by intravenous injection of heparin. Injection of heparin in lipemic subjects caused a small rise in NEFA concentration of plasma, but quantity of triglyceride hydrolysed was insignificant in relation to its large turnover rate.

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Successful Replacement Therapy in Lactating Thyro-parathyroidectomized. Rats.* (25746)

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Successful replacement therapy in thyro-parathyroidectomized (TPEC) rats requires optimal amounts of thyroxine (T4) as well as parathyroid hormone (PTH). Study of T4 secretion rate in lactating rats indicated 3 μg/100 g body weight (BW) was upper normal range(1). When this amount was injected into lactating rats from days 7-13 postpartum, milk yield increased 37% and when maximum removal was insured with oxytocin (OXT) milk yield increased 63.2%(2). With T4 and PTH at levels of 10, 2 x 20 and 3 x 20 USP parathyroid units/day partial replacement therapy of TPEC lactating rats was effected(3). Completely successful replacement therapy is here reported using both litter weight and milk yield on day 14 as indices. Technics are described by which animals with accessory parathyroid tissue (APTT) may be detected.

Material and methods. Primiparous lactating rats of Sprague-Dawley-Rolfsmeyer strain were housed in individual cages, fed Purina Lab Chow and water *ad lib.* Animal room was maintained at uniform temperature of 78 ± 1°F. Shortly after delivery, each litter was reduced to 6 young. TPEC was performed under ether anesthesia in less than 20 minutes on day 7 postpartum (PP). Replacement therapy was started shortly after operation: 3 μg T4/100 g/day and 2 x 40 USP units PTH‡/day were given subcutaneously. Parameters of lactational performance were mean litter weight and milk yield. Amount of milk removed by litter of 6 on day 14 under the influence of 1 USP unit oxytocin (OXT)§ was estimated by method described previously(4). To repeat this test at 24 hour intervals litters were weighed at end of 10 hours isolation, immediately replaced and allowed to nurse for 30 minutes. Litters were weighed again to nearest 0.5 g. Difference in litter BW before and after each nursing was used to represent amount of milk secreted during previous 10 hours. After this test, litters were allowed to stay over-night with mothers. To prevent feed intake other than mother's milk by young, mothers were allowed to eat only during 10 hour separation period from day 15 on. PTH was discontinued 5 hours prior to nursing period in 22 animals on day 14, in 12 animals morning of day 13.

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‡ Parathyroid hormone kindly supplied by Eli Lilly & Co., Indianapolis, Ind.

§ Oxytocin supplied by Armour Lab., Chicago.

Results. TPEC rats given replacement therapy of 3 μg T4/100 g/day and 2 x 40 USP units PTH/day from day 7-14 of lactation raised litters of 6 young successfully, reaching normal litter weights although weight gain from day 6-13, 14 was lower because of extremely high litter weights on day 6 (Table I). Similarly, lactational performance on day

TABLE I. Successful Parathyroid Hormone Replacement Therapy in Thyro-parathyroidectomized Rats.

*	Treatment (T4, PTH: Day 7-14, 15; OXT: Day 14, 15)	No. of rats	Killed on day	Day 6 total, g	Mothers' avg BW Day 14 Total, g	Mothers' avg BW Day 14 Gain or loss, %
A.	Control, no treatment†	30	14	297	300	+ 1.1 ± .4
B.	Control‡ T4: 3 μg/100 g/day, day 7-13 OXT: 1 USP unit, day 14	30	14	280	286	+ 2.1 ± .4
C.	TPEC: Day 4§ T4: 3.5 μg/100 g/day, day 4-14 PTH: None OXT: "	8	14	271‖	240	−11.0 ± 3.2¶
D.	TPEC: Day 7 T4: 3 μg/100 g/day, day 7-14 PTH: 2 × 40 USP units/day, day 7-14 OXT: 1 USP unit	10	14	296	275	− 7.3 ± 1.0
E.	TPEC: Day 7 T4: *Idem* PTH: " , day 7-12; 40 USP units day 13 OXT: None Died in tetany	10		283	(7) 242	−12.1 ± 1.6
F.	TPEC: Day 7 T4: *Idem*, day 7-15 PTH: *Idem* OXT: 1 USP unit, day 14, 15 APTT: Survived	2		276	273	− .9
G.	TPEC: Day 7 T4: *Idem* PTH: " , day 7-14 OXT: *Idem* 15 cases tetany, 13 fatal	15		276	271	− 2.0 ± .8
H.	TPEC: Day 7 T4: *Idem* PTH: " OXT: " APTT: Survived	7		282	272	− 3.5 ± 1.6

*	Litters' avg BW Day 6 Total, g	Day 13 Total, g	Day 13 Gain, %	Day 14 Prenursing total, g	Day 14 Gain, %
A.	79			164	112 ± 2
B.	74			166	124 ± 1
C.				93	
D.	86 ± 3[1]	173 ± 5	100 ± 5[5]	171 ± 4	99 ± 5[9]
E.	88 ± 2[2]	159 ± 4	(9) 80 ± 4[6]		
F.	84	168	99	173	106
G.	89 ± 2[3]	167 ± 4	88 ± 3[7]	166 ± 4	87 ± 3[10]
H.	81 ± 2[4]	171 ± 6	110 ± 6[8]	173 ± 6	113 ± 5[11]

(Continued on next page)

TABLE I (continued).

*	Avg milk yield: Day 14 — Total, g	Day 14 — Total as % of postnursing LW	Day 14 — Increase over normal, %	Day 15 — Total, g	Day 15 — Total as % of postnursing LW
A.	6.2	3.8 ± .3			
B.	11.0	6.2 ± .1	63		
C.	1.6 ± .4	1.7 ± .3			
D.	10.6 ± .7	5.8 ± .2	53		
E.	No milk yield				
F.	8.0	4.4	18	13.0 (11)	6.9 (11)
G.	10.2 ± .4[12]	5.8 ± .2[14]	53	12.2 ± .8[16]	6.8 ± .4[18]
H.	10.6 ± .8[13]	5.7 ± .3[15]	50	13.1 ± .8[17]	7.0 ± .3[19]

* In the second and third parts of the Table, letters alone (A, B, C, etc.) are used for treatment, No. of rats and day killed.

† Data from Grosvenor & Turner(5). ‡ Data from Grosvenor & Turner(2). § Data from von Berswordt-Wallrabe & Turner(3). ‖ Day 4. ¶ Day 4-14.

Figures in parentheses = No. of rats surviving, healthy.

APTT, accessory parathyroid tissue; TPEC, thyro-parathyroidectomy; BW, body wt; LW, litter wt; OXT, oxytocin; PTH, parathyroid hormone; T4, l-thyroxine.

Student's "t" test

	p value		*p value*
6–8		5–7	
7–8 10–11	$<.001$	12–16 14–18 15–19	$.01 < P < .025$
5–6	$.001 < p < .005$	2–4	
		3–4 13–17	$.025 < p < .05$

14 aided by OXT was comparable to milk yield of normal rats administered T4 plus OXT.

To determine completeness of operation, PTH was withdrawn on day 13 in 1 group. Ten of 12 animals had fatal attacks of tetany within 6 to 18 hours which caused depressed body and litter weights. These animals were not separated from young. On day 14 mothers of a second group were separated from litters for 10 hours for milk yield test. Last PTH injection was given 5 hours before this test. Of 22 animals, 13 had fatal attacks of tetany and 2 others severe attacks between days 15 to 18. Animals free of tetany until day 18 were assumed to possess APTT. Of the group of completely parathyroidectomized rats, milk yield test was repeated on day 15. In 4 animals showing heavy signs of tetany, only 6.2 g milk was produced whereas in 11 others, milk yield was significantly greater than on day 14. The same was true of group of rats with presumed APTT.

All operated mother rats lost body weight during lactation in contrast to normal lactating rats which gain slightly.

Discussion. These data demonstrate that PTH plays an important role in the lactation process. Limitations in amount of hormone administered reduced capacity of rats to secrete milk normally(3) whereas with 2 x 40 USP units/day lactation levels higher than those of normal rats were attained. This was made possible by concurrent administration of T4 (3 μg/100 g/day). Lactation sustained by replacement therapy was actually in excess of that which might have been produced by animals on lower level of T4.

Amount of PTH which successfully maintained lactation may be considered tentatively as estimated equivalent PTH secretion rate of this strain of lactating rats (29-30 USP units/100 g/day).

Although discontinuation of PTH in 1 group on day 13 proved that successful replacement therapy was achieved, it was not possible to run a test for milk yield on day 14, because all animals which did not have APPT

succumbed to tetany in less than 24 hours, whereas at lower levels withdrawal of PTH did not generally result in fatal attacks of tetany by this time(3). Another group of animals was injected until day 14 with PTH. Young were separated from mothers for 10 hours and milk yield was determined. Survival time of mothers was considerably prolonged and milk yield on day 15 obtained by same technic was significantly higher in animals not yet affected by tetany. Increased survival time may be explained by gradual inhibition of lactation and corresponding reduction in blood Ca removal during 10 hour separation period, whereas in previous group, young nursed continuously. More difficult to explain is the mean increase in milk yield on day 15 as compared to day 14, 29 hours after last injection of PTH. The increase was observed even in animals which then died in tetany a few hours later.

Summary. Thyroparathyroidectomy was performed in 44 lactating rats on day 7 postpartum. Successful replacement therapy was started immediately after surgery with optimal level of T4 (3 μg/100 g/day), 2 x 40 USP units PTH/day (corresponding to 29-30 USP units/100 g/day) and 1 USP unit OXT for complete milk removal on days 14-18. Litter weight and milk yield on day 14 reached levels of normal animals given optimal amounts of T4 (3 μg/100 g/day) and 1 USP unit OXT for milk yield test. Discontinuation of PTH on day 13 resulted, within 6-18 hours, in 10 fatal attacks of tetany in a group of 12. Withdrawal of PTH on day 14 coincided with 10 hour isolation period for milk yield test. A group of 15 such animals survived considerably longer than previous group, due, it is believed, to gradual inhibition of lactation and reduction in blood Ca removed during separation period. In 11 rats which succumbed to tetany later, milk yield was significantly higher on day 15 as compared to day 14, 29 hours after last PTH injection. Nine rats which lactated normally without exhibiting signs of tetany during 4 day period after withdrawal of PTH, were considered to have (accessory) parathyroid tissue. It is tentatively proposed to consider 29-30 USP units of PTH/100 g/day as estimate of parathyroid secretion rate in this strain of intensively lactating rats under beneficial influence of optimal T4 level.

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Non-Obstructive *Escherichia coli* Pyelonephritis in the Rat.* (25747)

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Pyelonephritis has not resulted from intravenous injection of coliform organisms in the experimental animal, in most reported studies, unless preceded by renal injury(1,2,3,4). A "virulent" strain of *E. coli* previously described(5) will, however, produce renal infection in some normal rats when injected intravenously. Pyelonephritis produced by *E. coli* tends to run a self-limited course of only a few weeks even though damage and inflammation are said to persist after infection has terminated(6). The course of pyelonephritis in the animal has been assessed by postmortem cultural and histological methods and, in some instances, by infrequent urine samples obtained by laparotomy and needle aspiration of bladder(3,6,7). "Clean-voided" urine collections with quantitative urine cultures have come into widespread use for evaluation of

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FIG. 1. Method of collecting urine specimens.

urinary infection in the human, but their use in the experimental animal has not been reported. The present investigation studies the course of non-obstructive pyelonephritis in the rat utilizing strain of *E. coli* mentioned above and "clean-voided" urine specimens.

Methods. Twenty-nine white, female Sprague-Dawley strain rats, weighing 100-140 g, were used. During control period each was found to have normal blood pressure and negative urine by microscopic examination and culture. Twenty-one experimental animals received approximately *100 million bacteria intravenously initially* and 12, which failed to develop bacteriuria of significant degree (>100,000/ml), received an additional similar injection 4 weeks later. Eight rats served as uninjected controls. Urine collections were made at 2-week intervals. Specimens were obtained in sterile test tubes after removal of lower abdominal fur with clippers and thorough cleansing of urethral papilla with 70% alcohol. Application of 300 volts by means of electrical stimulator† resulted in passage of urine in most instances, if animal had not been previously disturbed. On occasions when a specimen was not obtained, an attempt was usually successful on the following day. Method of collection is illustrated in Fig. 1. Culture of area surrounding urethral papilla, after above preparation, was carried out in all animals on one occasion as a control measure and while yielding *Staphylococcus aureus* or *albus* in a few animals, did not yield Gram negative bacteria. Urine specimens were studied microscopically, tested for protein, and cultured in thioglycollate broth and on eosin methylene blue, desoxycholate and blood agars. When growth resulted, bacteria were subcultured in citrate broth and on triple sugar iron agar for further identification. When volume of urine sufficed, serial dilutions of urine in desoxycholate pour plates were incubated and colony counts made. Determination of weight and blood pressure were also made every 2 weeks. Urine osmolarity was determined cryoscopically using Fiske osmometer and technic described by Hollander(8). Preparation of intravenous inoculum, methods for cultural and histological study of kidneys at time of sacrifice, and method of blood pressure determination have been described(5). At time of sacrifice blood was also obtained from the aorta for determination of blood urea nitrogen, and the bladder excised and placed in formalin for histological study.

Results. Significant results are shown in Table I. Of the 21 rats injected, one died of pneumonia after 17 days, and 7 failed to show cultural or histological evidence of urinary infection. The 8 uninjected controls were likewise normal throughout. Therefore, the data for these 16 animals are not presented in the Table. There was no significant difference in body weight, blood urea nitrogen, blood pressure or urine osmolarity between experimentals and controls. This is not surprising since marked renal destruction was not produced. Illustrations of microscopic lesions encountered are shown in Fig. 2. All lesions were focal and many were minimal. Microscopic evidence of cystitis was not found, but pyelitis and papillitis were encountered in 3 rats.

Of 13 experimental rats included in Table I, 3 had histological evidence of pyelonephritis, but urine cultures were only transiently positive early in the study. Of 10 with positive cultures of voided urine just prior to sacrifice, it was possible to obtain bladder urine by needle aspiration at time of sacrifice in 7 and all were positive for *E. coli*. Quantitative cultures of homogenized halves of each kidney

† Model 340 Stimulator, Harvard Apparatus Co., Dover, Mass.

were positive in only 2 of the 13, but broth culture of this material was positive in 5. Six had small, depressed cortical scars and 9 had evidence of pyelonephritis histologically.

Pyuria was present in the majority at some time during the 28-week period, but was neither striking nor persistent. Persistent proteinuria of significant degree occurred in only one (#15), and this rat had the most extensive disease.

Discussion. Persistent bacteriuria over a 7-month period is surprising in the light of the experience of others, but may be a feature of the strain of *E. coli* used which is unique in producing pyelonephritis in some normal rats. Presence of large numbers of bacteria in urine when they are no longer demonstrable in the kidney has been reported by other workers (7). The inconsistency between urine and kidney cultures may also be due to the fact that halves of 2 kidneys which were homogenized for culture may contain the only lesions, since they were focal, and similarly for the halves fixed for microscopic sections. Since it has been recently found that more severe infection in a larger percentage of animals can be produced after 2 or more "passages" of bacteria through rats with ligated ureters, further studies of the effect of nonobstructive *E. coli* pyelonephritis on blood pressure and renal function are planned.

TABLE I. Summary of Bacteriologic and Pathologic Findings at Time of Sacrifice (28 Wk).

Rat No.	Bacteriuria* colony count 10^6 in voided spec.	Bladder urine culture at sacrifice	Positive kidney culture	Pyelonephritis	
				Gross	Microscopic
1	+	+	+	+	0
2	+	+	+	0	+
5†	+	—‡	—‡	+	+
8†	0	0	0	0	+
10†	+	+	+	0	+
11	+	—	—	+	0
12	+	+	+	+	0
13†	0	0	0	0	+
15	+	—	—	+	+
18	+	+	0	0	+
20†	0	0	0	0	+
21	+	+	+	+	+
22	+	+	0	0	0

* Included on basis of 2 specimens prior to sacrifice, though most during study period were positive.
† Received second inj. of 100 million *E. coli*.
‡ Not obtained.

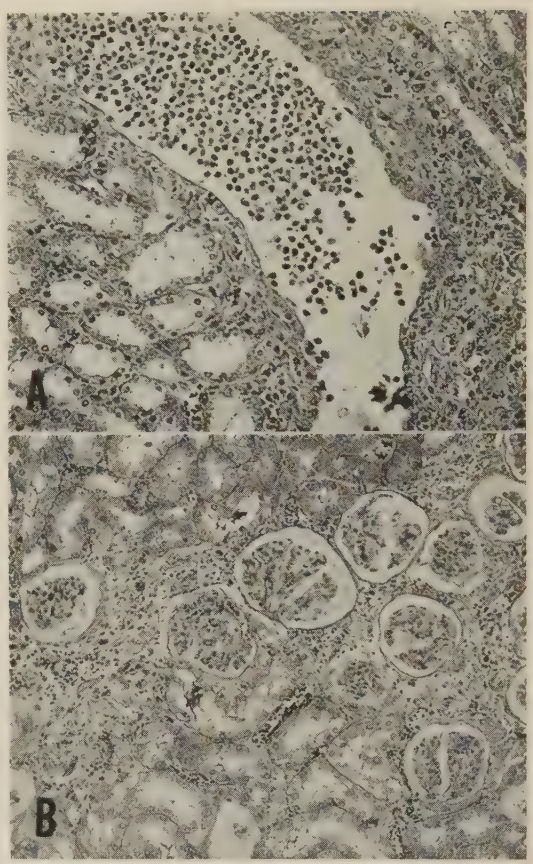

FIG. 2. Illustrations of microscopic lesions encountered. A. Chronic inflammation in wall of renal pelvis. Lumen contains polymorphonuclear leukocytes (×200). B. Scar in renal cortex of Rat 15 (×150). Hematoxylin and eosin.

Summary. An experimental model for study of non-obstructive *E. coli* pyelonephritis in the rat and successfully employing use of "clean-voided" urine specimens for quantitative culture has been described. It appears that some strains of *E. coli* may persist for long periods in the urinary tract and that minute lesions may be responsible for bacteriuria.

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Response of Plasma NEFA Levels to Epinephrine Infusions in Normal and Obese Women.* (25748)

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The physiologic and pharmacologic behavior of adipose tissue within the human body may well be a major etiologic factor in human obesity. It has been demonstrated that epinephrine markedly enhances release of non-esterified fatty acids (NEFA) by adipose tissue both *in vitro*(1) and *in vivo*(2,3). There is much evidence that nor-epinephrine, secreted by post-ganglionic sympathetic fibers, and epinephrine derived from the adrenal medulla are important regulators of fatty acid release from tissue fat depots(4). The present study was undertaken to test the hypothesis that obese subjects might exhibit a gross quantitative difference from normal individuals in response of serum NEFA levels to infused epinephrine.

Methods. Ten obese and 9 control female subjects were selected from out-patient department or hospital staff. All were free from complicating disease, had no personal or family history of diabetes, and were normally active on an ambulatory basis. Each was instructed to eat freely on day prior to infusion, but no food was allowed after 7 p. m. At 8 a. m. the following morning (13 hr. fast) a control blood sample was drawn at time of insertion of the indwelling needle. Isotonic saline was infused for 30 minutes (control period), whereupon a second sample was obtained. After control period, epinephrine bitartrate was added to infusion bottle to produce an infusion rate of 0.01 μg/lb/min (as epinephrine equivalent) and the infusion continued 30 minutes. At termination of the infusion (while epinephrine was still being infused) a third sample was taken. All blood samples were added to pre-chilled oxalate tubes, put in packed ice, centrifuged at 4°C, and immediately extracted and titrated for NEFA by Dole's method(3). An occasional sample was frozen at –15° for 1-2 days before

TABLE I. Response of Plasma NEFA Levels to Epinephrine Infusion* in Normal and Obese Women.

		NEFA conc. (μEq/l)			Glucose conc. (mg %)		
Age	Wt	C_1†	C_2‡	Epin.§	C_1	C_2	Epin.
		Normal women					
23	110	392	507	1209	87	90	108
17	115	586	682	1045	91	86	99
23	123	739	721	1243		75	81
23	129	564	691	1061	89	89	106
28	130	605	722	913	80	83	90
37	130	485	376	664	83	80	84
22	133	603	588	1102	101	96	119
25	135	715	885	1875	89	80	91
23	140	374	456	900	97	96	109
‖ 26	127	562	625	1112	90	86	99
		Obese women					
22	144	694	900	1780	93	90	125
21	185	608	681	1108	100	94	125
40	188	555	748	1676			
30	190	429	424	755	85	86	106
20	198	366	466	805	90	90	119
20	210	391	355	753	96	96	134
25	215	590	648	1242			
31	235	535	583	1055	91	93	111
20	300	676	746	807	88	84	127
39	337	885	980	1173	94	92	117
‖ 27	220	573	653	1115	92	91	121

* Infusion rate = 0.01 μg epinephrine/lb/min., 30 min.
† Initial control value.
‡ 30 min. " " .
§ At end of epinephrine infusion.
‖ Mean value.

* This study carried out during tenure of traineeship. Supported by Grant by Nat. Inst. of Arthritis and Metabolic Diseases, U. S. Public Health Service.

analysis. Plasma glucose determinations were also done on all samples.

Results. Infusion of epinephrine at the rate of 0.01 μg/lb/min produced objective symptoms (restlessness and tachycardia) in only one patient, and most subjects were unable to note any subjective difference from the control period. Mean fasting values in normal and obese patients were nearly identical in this series of patients (Table I). It has previously been noted, however, that obese patients tend to have higher fasting NEFA levels than normals, although variable overlapping between the 2 groups has been present(3,5). Mean fasting value in normals in the present study is nearly identical with that reported by Munker in a series of 107 normal patients(5). The rise in plasma NEFA levels following epinephrine infusion ranged from 191-990 μeq/l in normals, and from 61-1086 μeq/l in overweight individuals. Mean rise and final level attained in each group was identical within the limits of the method. Plasma glucose levels tended to increase much more following epinephrine infusion in the obese group (avg. 30 mg %) than in normal controls (avg 13 mg %), a difference which has statistical significance ($p<0.001$).

Conclusion. These observations suggest, then, that there is no impairment in ability of fat stores in obese patients to release NEFA in response to epinephrine.

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Mydriatic Half-Life of a New Anticholinergic as Affected by Dose, Route, Quaternization.* (25749)

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It has long been recognized that clearance of many drugs from the bloodstream approximates an exponential relationship with time (1-4), presumably because rate of clearance depends upon concentration of drug in blood. By extension, if degree of pharmacologic response and its rate of disappearance are determined by concentration of the drug on receptor sites, then disappearance of response should also approximate an exponential curve. The negative reciprocal of the slope of this semi-log curve would be the biologic half-life, a very useful index of drug performance (5). Recently, we determined the influence of dose, route, and quaternization on mydriatic half-life in mice of a new tertiary ammonium anticholinergic drug, SKF 5515(6).

Methods. Adult, male, Dierolf mice, in groups of 9-12, were given varying doses i.v., s.c., or p.o. of SKF 5515, 9-methyl-3-oxo-9-azabicyclo-(3,3,1)-nonan-7-yl benzilate maleate(6), or of SKF 5516, the methobromide quaternary derivative, and were observed at intervals for mydriasis. Pupillary diameter was measured in arbitrary units (5 units ≅ 1 mm) under constant illumination, using ocular micrometer. At each interval, the geometric mean increases in pupil size for each group were plotted on semi-log graph paper and least-squares lines fitted to a linear portion of the decay curve. The least-squares equations were in the form of $M = a\ e^{-kt}$, where M = change in pupillary diameter in units, a = a constant determining the extrapolated ordinate intercept, t = time in hours, k = a velocity constant in hr^{-1}, and e is the Naperian base. Apparent half-lives ($t_{½}$) in hours were then calculated(5) from $t_{½} = 0.693/k$. The "total drug effectiveness," *i.e.*, areas under time-action curves (F), were cal-

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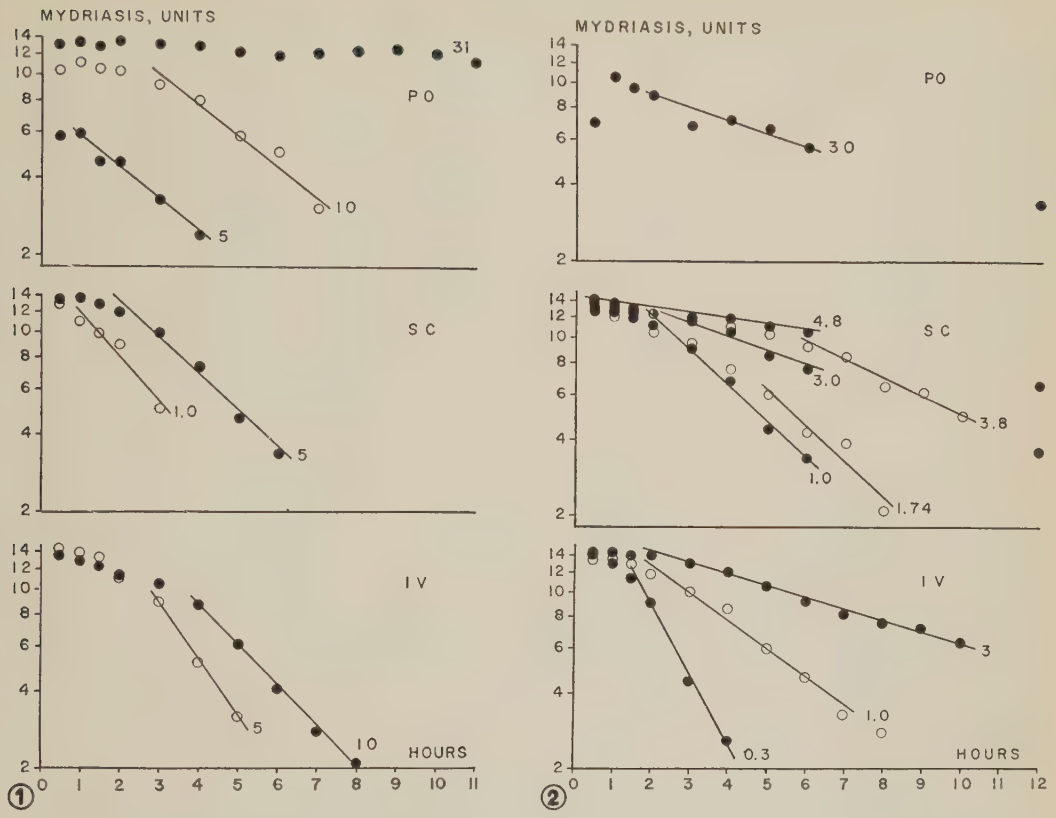

FIG. 1. Time-action curves for SKF 5515-induced mydriasis in mice. Ordinate: geometric mean change in pupillary diameter in arbitrary units; logarithmic scale. Abscissa: time after treatment in hr. Numbers adjacent to curves denote dosage in mg/kg. No. of animals/group given in Table I.

FIG. 2. Time-action curves for SKF 5516-induced mydriasis in mice. Other notes as in Fig. 1.

culated according to Dost's formula† (7) as

$$F = \int_0^\infty f(t)dt = a/k,$$

in terms of unit-hours.

Results. The effect of highest oral dose of SKF 5515 was not followed for a sufficiently long time to observe disappearance of mydriasis (Fig. 1). For other routes and doses of SKF 5515, disappearance appeared exponential. The harmonic mean half-life, weighted by number of mice and observations/group, was 2 hr (Table I). Comparison of F-values for the same doses given by different routes reveals a reasonably efficient absorption of the drug as the number of absorptive barriers is increased, *i.e.*, as route changes from i.v. to s.c. to p.o. SKF 5516 was poorly absorbed orally (Fig. 2, Table I). In fact, at highest dose, 50 out of 60 mice displayed no mydriasis; the remaining 10 were pooled and graphed. Unexpectedly, the apparent half-life following i.v. or s.c. treatment changed with dose level: $t_{1/2}$ was roughly correlated with log dose. Comparison of F-values for SKF 5516 shows little difference between parenteral routes. Comparison between SKF 5515 and SKF 5516 suggests a greater parenteral effectiveness of the latter, attributable in part to dependence of $t_{1/2}$ on dose. Orally, of course, the tertiary compound is much more effective. Nothing can be said of the relative mg potencies of the 2 compounds, because, under this experimental design, almost all

† Although Dost proved this relationship for single and double consecutive first-order reactions, it can be shown by mathematical induction to hold in general for *n*-consecutive reactions.

TABLE I. Mydriatic Responses.*

Drug	Route	Dose, mg/kg	No. mice	Total No. observations	α	k	$t_{1/2}$	F
SKF 5515	i.v.	5	12	36	43.4	.53	1.3	80
	"	10	10	50	36.7	.36	1.9	100
	s.c.	1	"	40	17.9	.40	1.7	45
	"	5	"	50	25.3	.33	2.1	75
	p.o.	5	"	"	7.7	.28	2.5	25
	"	10	"	"	22.2	.27	2.6	80
	"	31	"		Not mensurable			
SKF 5516	i.v.	.3	12	48	30.5	.62	1.1	50
	"	1.0	"	72	20.5	.25	2.8	80
	"	3.0	"	108	17.7	.10	6.6	170
	s.c.	1.0	10	50	22.9	.32	2.2	70
	"	1.74	"	40	31.8	.33	2.1	100
	"	3.0	"	50	17.1	.13	5.3	130
	"	3.8	"	"	26.9	.17	4.1	160
	"	4.8	9	72	14.2	.045	15.3	315
	p.o.	4.8	10		No effect			
	"	10.0	"		"	"		
	"	30.0	10/60	70	11.2	.11	6.2	100

* See text for definitions and units of α, k, $t_{1/2}$, and F.

doses caused maximal mydriasis.

Discussion. If we assume that true mydriatic half-life of both drugs is approximately ½ hr, then the apparent increase seen with increasing doses of SKF 5516 suggests that the data to which logarithmic curves were fitted had not yet become truly exponential. This is, of course, an inherent danger in fitting physical curves to biologic data(1). In the present case, we may theorize that kinetic reactions before or after pupillary reaction are responsible, *e.g.*, an initial sequestering of SKF 5516 in peripheral tissues coupled with a slower release of the drug therefrom, a decreased penetrability of absorptive barriers resulting in penetration rates less than that expected on a monomolecular basis, or a concentration-dependent inhibition of drug removal from the eye. It is well-known that mydriasis itself physically impedes drainage of the aqueous humor by compression of the Canal of Schlemm, and that, therefore, mydriasis lasts longer than peripheral anticholinergic effects(8). This, however, cannot explain the present results, because the half-life of SKF 5515 was independent of dose and route. If we assume that the passage of SKF 5516 across absorptive membranes was hindered by the ionic charge and that ante-pupillary kinetic reactions were involved, the data become explicable, even though our assumptions may be incorrect or incomplete. Naturally, the situation is further complicated by the greater ganglion-blocking potency of SKF-5516.

The foregoing thoughts have been based on the assumption of a first-order disappearance of pharmacologic response. This may not be exactly true even though our data may fit such a hypothesis. As has been shown by others(9-11), the drug-receptor reaction may theoretically and experimentally be described by a reversible second-order reaction. In this case, if the dissociation constant for the drug-receptor complex(9) is sufficiently large in comparison to first-order rate constant for detoxication/elimination, then disappearance of pharmacologic activity will rapidly approximate the exponential curve determined by the latter constant. On the other hand, if the dissociation constant is not sufficiently large, the disappearance curve will depart from exponentiality and this departure may become detectable graphically or it may be inferred thermodynamically(10). In general, however, pharmacologic data are obtainable with insufficient accuracy to allow exact description of the underlying kinetic reactions, thus forcing us, for practical purposes, to accept data that appear to fit the simplest assumption, *viz.*, first-order disappearance, with the realization that this is in the nature of an approximation.

Realizing that a drug may have not only different affinities and intrinsic activities for different target organs(9), but, reciprocally and as a result thereof, also different apparent

half-lives, we endeavored to quantitate the time-action curves of these 2 drugs using tachycardia and salivary-suppression in mice. However, difficulties of obtaining precise time-action curves led us to abandon these experiments and to pursue our studies of these 2 drugs using different species. Current approaches also include anticholinergic Q_{10}'s in poikilotherms and simulation of experimental curves on an electronic analog computer.

Summary. The mydriatic half-life in mice of SKF 5515, a new tertiary ammonium anticholinergic drug, was independent of dose and route within the limits of experimental error. The apparent half-life of the methobromide quaternary derivative was dependent upon log dose and route, possibly as a result of decreased rate of penetration of membrane barriers by the charged ion.

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Comparison of Heparin Induced Lipid Clearing Activity in Human Thoracic Duct Lymph and Plasma.* (25750)

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Plasma from humans and other species after intravenous heparin administration (post-heparin plasma) when incubated with lipemic serum or milky lymph, produces a decrease in optical density, or "clearing" of fat(1). Rate of decrease in optical density (clearing activity) of such lipemic substrates has been used as index of lipoprotein lipase activity in post-heparin plasma(1,2,3). We tested for clearing activity in human thoracic duct lymph to compare it with that in plasma of the same individual.

Methods. Five patients were studied. Two had disseminated cancer, 2 had Laennec's cirrhosis, and the 5th had dyspeptic symptoms following cholecystectomy 3 years previously. The thoracic duct was cannulated in the neck by technic of Linder and Bloomstrand(4). The observations were made on 1st or 2nd post-operative day. Samples of plasma were collected before and 5 and 10 minutes after administration of 10 mg of heparin to 2 patients and 50 mg to the other 3. Consecutive 10 minute samples of lymph were collected continuously before, during and after heparin administration. Furthermore 3 received intravenous heparin 1 day prior to operation, and these post-heparin plasma samples were assayed for clearing activity. Specimens were collected in chilled test tubes containing oxalate and were kept in ice bucket until centrifuged at 30,000 rpm for 15 minutes in refrig-

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erated centrifuge and then stored at –4°C. Clearing activity was determined by turbidiometric method as modified by Robinson and French(5,6). A stock supply of human milky lymph was used as substrate; this was stored frozen following its collection from a patient 3 months previously, and diluted 1:100 with physiological NaCl solution to give optical density readings of less than .7 in Bausch and Lomb colorimeter (Spectronic 20). Plasma and lymph samples were thawed at 4°C and incubated at 37°C in water bath 5 minutes. Six cc of substrate (also incubated at 37°C for 5 minutes) were then added to 1 cc of sample tested and optical density read immediately at wavelength of 640 μ in the colorimeter. Tubes containing the mixture were then returned to water bath and readings made at 15 minute intervals for 1 hour. One tube of substrate without plasma was used as control and a tube of saline as blank. Clearing activity was expressed in units calculated as amount of decrease in optical density/minute.

Results. In all 5 patients, only plasma samples collected after heparin administration promoted clearing activity. There was no clearing activity in thoracic duct lymph *in vitro* or *in vivo*.

In 3 patients whose plasma response was determined both before and during duct cannulation, the same degree of clearing activity was demonstrated at both times. Samples of lymph collected 20 and 30 minutes after heparin administration failed to demonstrate clearing activity. Results in 1 patient, which are characteristic of the others, are shown in Fig. 1.

Discussion. Inability to demonstrate clearing activity in human thoracic duct lymph after administration of heparin in quantities sufficient to cause appearance of this activity in plasma is not in accord with results reported by Young and Freeman in rats and rabbits(7). This discrepancy is perhaps due to differences in amount of heparin used, since on a weight basis the animals received at least 10 times the dose employed in these patients.

Robinson and French(8) reported that ligation of thoracic duct in rats increased amount of clearing activity in post-heparin

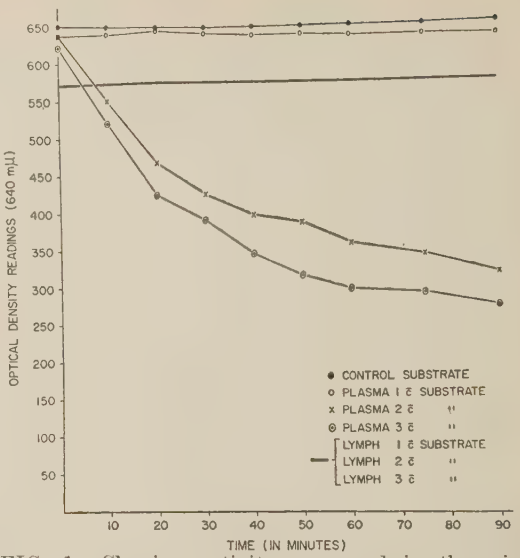

FIG. 1. Clearing activity compared in thoracic duct lymph and in plasma after heparin administration. Results in this patient are representative of results in the other 4.

plasma. They suggested that this increase represented decreased utilization of clearing factor *in vivo* when fatty chyle is prevented from entering the blood. Although thoracic duct was not ligated in our experiments, complete diversion of thoracic duct lymph did not change post-heparin plasma clearing in 3 patients tested before and during cannulation. It seems unlikely therefore that the explanation offered by Robinson and French is correct.

Heightened plasma clearing response to heparin has been reported in patients with chronic liver disease(9). Two patients with cirrhosis of the liver had no different response in either plasma or lymph from the other 4 patients.

Rate of decrease in optical density of a substrate consisting of milky lymph, when incubated with samples of either lymph or plasma, has been used in these experiments as assay for clearing activity. No determinations were made of changes in free fatty acids or neutral fat in the incubation mixture. Others have shown however, that *in vitro* decrease in optical density, or clearing, comes about through formation of soluble fatty acid-protein complexes with concomitant reduction in triglycerides(10).

Summary. *In vitro* clearing activity has been measured in samples of human plasma and thoracic duct lymph before and after heparin administration. When administered in amounts sufficient to cause lipid clearing activity in plasma, heparin does not produce clearing activity in thoracic duct lymph.

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Depot Fat Mobilization in Spinal Cord Sectioned and Restrained Rats.* (25751)

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The work of Goering(1), Wertheimer(2), Hausberger(3), Boecke(4) and others supports the concept that metabolism of adipose tissue is influenced by neural factors. Thus, autonomic nervous system may alter movement of fat to and from storage depots(3, 5-8). Our previous work showed that certain autonomic blocking agents prevented mobilization of fat from epididymal depots of adrenalectomized rats during fast. Surgical intervention within the nervous system eliminates ambiguities of the mechanism of blocking drug action. Therefore, spinal cord section was used as alternative tool. Since paralysis induced by this procedure alters activity of animal and the energy expended, restraining cages were used to immobilize equally both control and cord sectioned animals.

Methods. Male rats of Holtzman strain, weighing 90-110 g, were used. The epididymal fat pad was utilized as index of peripheral depot mobilization. Fat body removal was performed as previously described(9). Total lipids were extracted and weighed according to Bloor's method(10). The first fat depot was removed just prior to beginning of 24-hour fast. The second pad was excised upon termination of starvation. The difference between amount of extracted fat in the 2 epididymal depots determined extent of fat mobilization. Initial depot removal was altered from side to side throughout experiments. All spinal cord sections and sham operations were performed under ether anesthesia. A midline incision was made over region of thoracic vertebrae 2-4. Laminectomy was performed and spinal cord exposed, which was then severed. Muscles were sutured and skin closed with wound clips. Only those animals with complete cord transection, as determined on autopsy, were used. Sham operations consisted of cord transection procedure without actual cutting of the spinal cord. Fasting began with end of surgery. Restraint was accomplished by confining the rat within a wire screen cylinder. This prevented most bodily activity and allowed only slight movement of head. Restraint immedi-

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TABLE I. Effect of Spinal Cord Transections (T_2–T_1) and Restraint on Body Weight and Epididymal Fat Mobilization during a 24 Hour Fast.

Procedure	No. of rats	Body wt ± S.E.			Epididymal fat wt ± S.E.		
		Initial, g	Final, g	Diff., g	Initial, mg	Final, mg	Mobiliz., mg	Mobiliz., mg %
Intact	91	99.8 ± .21	82.6 ± .26	17.2 ± .12	108.9 ± .8	87.1 ± .9	21.8 ± .5	20.0 ± .4
Sham spinal cord section	23	97.4 ± .83	81.0 ± .57	16.4 ± .40	99.1 ± 2.8	71.5 ± 2.6	27.6 ± 1.6*	27.8 ± 1.6*
Spinal cord section	24	96.8 ± .60	88.8 ± .68	8.0 ± .36§	112.4 ± 3.6	81.2 ± 3.2	31.2 ± 2.5*	27.8 ± 2.6*
Intact + restraint	21	97.3 ± .77	78.3 ± .90	19.0 ± .41	93.7 ± 2.5	71.6 ± 2.6	22.1 ± 2.0	23.5 ± 2.0
Sham spinal cord section + restraint	23	98.7 ± .70	78.8 ± .83	19.9 ± .30	93.6 ± 2.5	51.2 ± 3.1	42.4 ± 3.3†	45.1 ± 3.4†
Spinal cord section + restraint	24	96.5 ± .63	90.6 ± .79	5.9 ± .47‖	106.3 ± 3.5	92.5 ± 2.6	13.8 ± 1.6‡	12.9 ± 1.7‡

* $p < .01$ compared to intact.
† *Idem*, sham cord section and intact plus restraint.
‡ *Idem*, spinal
§ $p < .01$ compared to all other procedures.
‖ *Idem*

ately followed any surgical procedure and marked the onset of fasting.

Results. Initial mean *body weights* were approximately the same in all procedures (96.5-99.8 g). Spinal cord sectioned animals lost significantly less body weight than did intact rats during the 24-hour fast. When lesioned rats were restrained, their body weight loss was even less than that observed due to cord section alone. However, sham cord section alone and with restraint, and restraint alone resulted in weight loss comparable to that demonstrated by unrestrained intact rats (Table I).

Fat mobilization. Since initial epididymal fat body weights varied (93.6-112.4 mg), amount of lipid mobilized is expressed not only as absolute weight lost but also as mg% initial fat weight. Amount of fat moved out of the depots during fasting period, was increased significantly ($p < 0.01$) over control values in both sham operated and cord sectioned animals. This is not consistent with total body weight changes. Rats which were restrained but did not have sectioned cords (intact or sham operated) showed about the same degree of fat mobilization as did control animals. Combination of sham operation and restraint resulted in marked increase of movement of lipids from these fat bodies ($p < 0.01$); whereas, cord section plus restraint produced significant inhibition of fat mobilization despite fasting state (Table I).

Discussion. Decreased loss of body weight in cord sectioned rats is not explained only by inactivity, since restraint alone in intact and sham operated preparations did not alter total body weight loss during this short fasting period. It is impossible, however, to determine relative amounts of energy expenditure using this crude technic. Smaller body weight loss of cord sectioned rats probably reflects mainly water retention.

The acute effect of either manipulation of spinal cord (sham) or actual transection augmented fat movement out of epididymal depots. Both procedures produce an immediate firing of peripheral nerve impulses. This may account for changes observed in fat mobilization in unrestrained animals.

On this basis, it is difficult to account for inhibition of fat mobilization in animals that were restrained after cord section and for pronounced increase in fat mobilization in sham operated restrained animals. Neural excitation initiated by manipulation of intact cord seems aggravated by muscle tension produced by restraint. This cannot occur in lesioned animals where muscle tonus is eliminated. Alteration in fat mobilization has been produced by these procedures and chronic experiments are planned to elucidate mechanisms involved.

Summary. Male rats were subjected to spinal cord section and restraint during 24-hour fast. Both sham operation and cord section increased movement of fat from epididymal depots. Sham cord section plus restraint resulted in even greater rate of lipid movement; whereas, section plus restraint inhibited fat mobilization.

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Fluorinated Pyrimidines XII. Effects of Simple Nucleotides on Transplanted Tumors.* (25752)

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Previous work demonstrated potent tumor inhibitory properties(1,2) of 5-fluorouracil (FU)(3) and its nucleosides, 5-fluorouridine (FUR) and 5-fluoro-2′-deoxyuridine (FUDR). Although it seemed improbable (4) that nucleotides could enter cells without breakdown, nevertheless the finding that 5 - fluoro - 2′ - deoxyuridine - 5′ - monophosphate (FUDRP) is a potent inhibitor of thymidylate synthetase(5,6) pointed to the desirability of testing this compound, as well as corresponding ribonucleotide, 5-fluorouridine-5′-monophosphate (FURP), against transplanted mouse tumors. FURP was prepared enzymatically by Dahl *et al.*(7), and chemically by Farkas *et al.*(8). However, the method of Gilham and Tener(9) appeared more convenient, and we have prepared gram quantities of FURP and FUDRP by this procedure. The synthesis will be described elsewhere, and we are greatly indebted to Dr. Tener for making the details available to us prior to publication. The 2 nucleotides have been tested at various dosages against 3 transplanted mouse tumors, and compared with equimolar amounts of the corresponding nucleosides. We are not aware of any published reports on tumor-inhibitory activity of nucleotides of purine or pyrimidine analogs.

Methods. Nucleosides, FUR and FUDR, were kindly supplied by Dr. Robert Duschinsky of Hoffmann-LaRoche, and were converted chemically into nucleotides, FURP and FUDRP(9). Female Swiss mice were obtained from A. R. Schmidt Co., Madison, Wis., and used for Sarcoma-180 and EF Ehr-

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TABLE I. Effect of Fluorinated Pyrimidine Nucleosides and Nucleotides on Growth of Sarcoma. Measurements are given for 14th day after transplantation. Dose administered intraperitoneally daily for 7 days, starting one day after transplantation.

Group	Dose, mg/kg/day	Wt change, g	Survivors, out of 6	T vol, mm^3	T/C
Controls	0	−1.3	5	920	
FUDRP	200	−4.7	2	15	.02
"	150	−2.3	3	38	.04
"	100	+ .5	6	54	.06
"	66.3	+2.6	6	257	.28
FUDR	50	−1.1	6	288	.31
FUDRP	30	+ .4	6	461	.50
"	15	0	6	929	1.01
"	10	−1.0	6	1240	1.35
"	5	−1.4	6	1850	2.02
Controls	0	−1.9	6	1420	
FUDRP	150	−2.7	3	15	.01
"	66.3	+ .3	6	50	.04
FUDR	50	− .6	6	21	.02
Controls	0	−1.4	6	967	
FURP	6	−5.6	4	954	.98
"	5	−5.5	5	936	.97
FUR	4.5	−7.8	5	781	.82
FURP	4	−5.1	6	914	.94
"	3	−4.6	6	644	.67

lich ascites carcinoma. L-1210 leukemia was carried in female BDF_1 mice from Cumberland View Farms, Clinton, Tenn. S-180 was transplanted bilaterally by trocar, and the 2 ascites tumors were transplanted intraperitoneally with suspensions of approximately 5 x 10^6 cells. Drugs were given once daily by intraperitoneal injection for 7 days, starting one day after transplantation. Survival of mice with ascites tumors was recorded, and in Sarcoma 180 experiments, tumors were measured periodically and volumes calculated(1). Because of limited supply of nucleotides, large numbers of mice or animals larger than mice could not be used.

Results. The effects of FUR, FURP, FUDR, and FUDRP against Sarcoma-180 are shown in Table I. Although there were only 6 mice in each group, there were actually 12 tumor measurements, since tumors were transplanted bilaterally. Although measurements were taken at various times, only data at 14 days after transplantation (one week after cessation of therapy) are shown here since they are representative also of other times. It is evident that FUDRP is a potent inhibitor of tumor growth from doses of about 30 to 150 mg/kg/day, although the latter dose is toxic. There are insufficient number of mice to justify calculation of a chemotherapeutic index. In 2 experiments 66.3 mg/kg of FUDRP and 50 mg/kg of FUDR, which are equimolar equivalents, are not significantly different in tumor-inhibitory potencies. Thus, the nucleotide does not appear to offer chemotherapeutic advantage over the nucleoside. On the other hand FUR and FURP, although of much greater toxicity than the corresponding deoxy compounds, produced no significant inhibition of this tumor. Even severe weight loss of mice had no effect on growth rate of S-180.

In marked contrast, as shown in Table II, FURP produced a significant prolongation of survival time of mice bearing Ehrlich ascites carcinoma. At doses of 3 and 4.5 mg/kg/day

TABLE II. Effects of Fluorinated Pyrimidine Nucleosides and Nucleotides on Survival of Mice Bearing Ehrlich Ascites Carcinoma. Dose conditions as before.

Group	Dose, mg/kg/day	No. mice	Avg survival, days	Extremes, days	100 day negatives	T/C
Controls	0	18	15.9	8- 19	0	
FUDRP	150	8	23.7	8- 35	0	1.5 Toxic
"	66.3	8	49.0*	18-100	2	3.1*
FUDR	50	8	33.5	11- 47	0	2.1
FUDRP	66.3(14 days)	8	56.0*	19-100	2	3.5*
FUDR	50 (")	8	41.0*	12-100	2	2.6*
FURP	4.5	10	50.9*	23-100	3	3.2*
FUR	4	10	9.8	8- 10	0	.6 Toxic
FURP	3	10	54.7*	16-100	3	3.4*
"	2	10	25.9	12- 85	0	1.6
"	1	10	18.6	12- 22	0	1.2

* Mice that survived 100 days were sacrificed, autopsied, and no tumors observed. Since it is impossible to calculate avg survival and T/C values under these conditions, values above were calculated as if the mice had died on 100th day. Thus these values are low.

TABLE III. Effects of Fluorinated Pyrimidine Nucleosides and Nucleotides on the Survival of Mice Bearing L-1210 Leukemia. Dose conditions as before.

Group	Dose, mg/kg/day	No. mice	Avg survival, days	Extremes, days	T/C
Controls	0	28	11.0	9-14	
FUDRP	150	8	11.2	10-13	1.0*
"	66.3	8	16.2	11-22	1.5
FUDR	50	8	15.6	9-22	1.4
FURP	6	20	15.3	9-21	1.4
"	5	20	15.2	9-28	1.4
FUR	4.5	20	12.2	9-20	1.1
FURP	3	20	20.1	12-48	1.8

* Toxic.

of FURP, 3 mice survived tumor-free for 100 days. At higher doses toxicity was prohibitive. It is interesting to note the very high toxicity of the nucleoside, FUR at dose, 4 mg/kg, equimolar to the highly therapeutic level of 4.5 mg/kg of FURP.

In Ehrlich ascites carcinoma, FUDRP appeared slightly more effective than FUDR at equivalent doses, and toxicity was low enough to allow treatment for 14 days, resulting in somewhat improved survivals.

In L-1210 leukemia (Table III) all compounds produced a moderate effect. FUDR and FUDRP appear of equivalent activity, and there is a suggestion that FURP may be somewhat more effective and less toxic than FUR.

Discussion. Although the number of mice in above experiments was small, necessitated by limitation in supply of nucleotides, some conclusions can be reached. In Sarcoma-180 and L-1210 leukemia FUDR and FUDRP had comparable activities, with the latter possibly more active in Ehrlich ascites tumor. On the other hand, in the 2 ascites tumors, FURP appeared less toxic and more effective than FUR, although neither compound exerted an effect on S-180. Further screening of FURP against other tumors is indicated. Whether possible advantage of FURP or FUDRP reflects penetration into cells or merely a different rate of excretion or body distribution is under investigation with suitably labeled compounds. In deoxy compounds the nucleotide provided no advantage over nucleoside. In practical terms, it would appear that before large-scale trials could be justified, a nucleotide would have to offer very significant therapeutic advantages over the corresponding nucleoside, which itself is expensive and difficult to obtain.

Summary. Tumor inhibitory activity of 5-fluorouridine-5′-monophosphate (FURP) and 5 - fluoro - 2′ - deoxyuridine - 5′ - monophosphate (FUDRP) have been studied in mice bearing Sarcoma-180, Ehrlich ascites carcinoma, and L-1210 leukemia. Comparisons have been made with equimolar equivalents of corresponding nucleosides, 5-fluorouridine (FUR) and 5 - fluoro - 2′ - deoxyuridine (FUDR). FUDR and FUDRP were inhibitors of S-180 of not strikingly different potency. FUR and FURP were inactive against this tumor. However, FURP at lower dose was as active as FUDRP against Ehrlich ascites carcinoma, producing about 30% 100 day survivors. FUR was extremely toxic. In L-1210 leukemia the compounds were of moderate and equivalent activity.

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Gonadotrophic Hormone Function in Persistent Estrous Rats with Hypothalamic Lesions.* (25753)

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Hypothalamic regulation of gonadotrophic hormone function in the rodent is well established but selective control over separate gonadotrophins has been difficult to assess. Destruction of the periventricular grey area and median eminence in the rat induces profound atrophy of female reproductive tract, a result which strongly suggests curtailment of both FSH and LH secretion by the adenohypophysis(1). In contrast, lesions in pre-optic and anterior hypothalamic areas produce persistent estrus, hypertrophied uteri and inhibition of corpus luteum formation in the ovary. Most experimenters consider these effects to reflect primarily a deficiency in LH secretion (2-5), with FSH-estrogen component of pituitary-ovarian interplay presumably spared. Others believe that destruction of the hypothalamic site governing reproductive rhythmicity abolishes an estrogen-sensitive area through which pituitary FSH secretion is cyclically restrained(6,7). Failure of feedback mechanism thus permits continued estrogen secretion from follicular ovaries. The present study attempts to resolve the basic problem of hormonal balance in persistent estrous lesioned rat by determining capacity of adenohypophysis to augment gonadotrophin secretion after unilateral oophorectomy. Degree of compensatory ovarian hypertrophy will be related to direct measurement of FSH in both blood and anterior pituitary.

Materials and methods. Young adult female albino rats (Wistar strain; initial weights 200-225 g) displaying 4-5 day cycles were used. Bilateral electrolytic lesions were made in pre-optic and anterior hypothalamic areas of the brain with Krieg-Johnson stereotaxic apparatus. Lesions were placed 7.0 mm anterior to ear plugs, .5 mm on either side of sagittal suture and .5-1 mm from base of skull. A direct current of 2 mA for 20 seconds was used. Lesion localization was checked on toluidine blue or chrome-alum-hematoxylin stained serial sections through the diencephalic area of formalin fixed, paraffin embedded brains. Persistent (frequent) estrus, determined from vaginal smears taken daily, was produced in approximately 75% of operated rats. Once well established (2-3 weeks after lesioning), periods of frequently occurring vaginal estrus, occasionally interspersed with 1 or 2 days diestrus, continued in some rats 4-10 months post-operatively. In the first experiment, 2 groups of estrous rats were unilaterally ovariectomized (left gonad) 25 and 60 days respectively after hypothalamic electrocautery. These rats, together with ovariectomized and intact controls, were sacrificed 19-21 days later. In the second experiment, vaginal cycles were studied for 120-300 days in a group of persistent estrous lesioned rats with gonads intact, to determine duration of effect and morphologic consequences to reproductive and endocrine systems. Just before autopsy, rats were lightly anesthetized with ether, and blood drawn by direct cardiac puncture. Sera obtained from pooled blood were frozen until time of assay. Right ovary, uterus (stripped of fluid), thyroids and adrenals were quickly dissected, weighed and prepared for histologic study. The hypophyses were rapidly removed. The anterior pituitaries, separated from posterior lobes by blunt dissection, were weighed to .1 mg on microtorsion balance and then pooled. Pituitary extracts for FSH assay were made by homogenization and extraction with acidified saline as routinely used for TSH(8). Sera and pituitary extract were assayed for FSH in 21 day old female rat using human chorionic gonadotrophin (HCG) augmentation method of Steelman and Pohley(9). The NIH-FSH-S1 preparation was used as standard.‡ Each

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TABLE I. Inhibition of Unilateral Compensatory Hypertrophy of Right Ovary in Hypothalamic Lesioned Persistent Estrous Rats.

Group	No. of rats	Mean body wt at sacrifice (g)	No. days lesioned	Mean ovarian wt (mg) Left	Right	% ovarian hypertrophy‡	Mean uterine wt (mg)
1st exp.							
Normal, intact	25	233 ± 3†	0	36.1 ± 2.4	40.4 ± 2.2	11.9 ± 5.7	491 ± 34
Normal, unilateral oophorectomy	25	250 ± 6	0	35.7 ± 1.5	57.4 ± 2.9	60.1 ± 8.0*	565 ± 41
Lesioned, unilateral oophorectomy	13	241 ± 7	44	30.2 ± 3.0	36.2 ± 4.2	19.9 ± 6.8	758 ± 46*
Idem	11	265 ± 10	81	33.3 ± 3.8	42.3 ± 5.9	27.0 ±10.0	611 ± 39*
2nd exp.							
Normal, intact	11	308 ± 12	0	42.7 ± 4.2	35.7 ± 2.8		659 ± 40
Lesioned, intact	9	312 ± 20	120–300	24.3 ± 1.9*	24.6 ± 2.0*		887 ± 63*

* Values considered significant ($P < .05$).
† Refers to mean ± stand. error of mean.
‡ Based on left ovarian wt (100%).

test animal was injected subcutaneously thrice daily for 3 days and received a total of 9 cc of serum or extract equivalent of 16 mg of anterior pituitary.

Results. Examination of the lesion in persistent estrous rats indicated a confluent area of necrosis basally placed and in the midline, extending rostro-caudally from pre-optic nuclear zone to supra-optic portion of the hypothalamus. Mid-regions of optic chiasm were frequently damaged, but supra-optic and paraventricular nuclei were usually spared. The caudal extreme of the lesion rarely impinged on the median eminence. Neighboring fiber tracts such as fornix and stria medullaris thalami were inconsistently affected. The tractus infundibularis was invariably damaged.

Three major findings are evident (Table I) namely; 1) left ovarian weight in lesion induced, persistent estrous rat remained within normal limits for at least 60 days post-operatively but eventually decreased. Histologically, the ovary appeared follicular in nature without evidence of fresh corpus luteum formation. 2) Despite eventual ovarian atrophy, significant uterine enlargement occurred in operated rats. 3) Compensatory hypertrophy of remaining gonad in unilaterally oophorectomized, lesioned animals was substantially inhibited. The degree of right ovarian hypertrophy (based on left gonad as 100%) in both groups of electro-cauterized rats was about 20-27%, values not significantly different from the normal right-left ratio (12%). Compensatory ovarian enlargement in rats not bearing hypothalamic lesions was 60%. There was no indication that lesions inducing estrus interfered with normal growth. Obesity rarely occurred. Although adenohypophyseal weight was not appreciably altered in short term operated animals, pituitary hypertrophy was found in rats still showing persistent estrus 4-10 months after hypothalamic destruction.

FSH assay of pituitary extract from unilaterally ovariectomized, lesioned rats indicated that ovarian response in test animals was slightly but consistently greater than that achieved with identical amounts of pituitary material from ovariectomized or intact controls (Table II). It thus appears that concentration of FSH in the adenohypophyses of lesioned rats following unilateral castration was at least equal to, if not greater, than in corresponding controls. Contrariwise, FSH was not detected in pooled sera from these animals but could be demonstrated in relatively high titer in blood of non-lesioned, castrates. (Detection of FSH in serum from intact control rats was questionable.) Although FSH content of blood and pituitary was not measured in persistent estrous animals with intact gonads, the results suggested that under conditions of enhanced demand, provoked by unilateral castration, FSH release from the pituitary was suppressed.

Discussion. Most investigators who have produced persistent vaginal cornification in rodents with hypothalamic lesions have also

TABLE II. FSH Assay of Adenohypophyses and Sera from Operated Rats.

Total dose FSH (μg)*	Mean ovarian wt (mg)	Donor group	Mean ovarian wt (mg) Adenohypophysis‡	Serum‡
0	24.1 ± 1.7†	Normal, intact	36.4 ± 3.7	31.2 ± 3.7
25	30.5 ± 5.1	Normal, unilateral oophorectomy	37.6 ± 4.6	39.4 ± 3.8
100	37.9 ± 3.8	Lesioned (44 days) unilateral oophorectomy	44.3 ± 2.2	27.2 ± 3.4§
250	84.6 ± 9.6	*Idem* (81 days)	43.2 ± 5.7	

* All bioassay test rats received 20 units of human chorionic gonadotrophin (courtesy of Dr. D. McGinty, Parke, Davis & Co., Detroit, Mich.).
† Refers to mean ± stand. error of mean based on 4-5 test animals.
‡ Test animals received extract equivalent of 16 mg of pituitary or 9 cc of serum.
§ Represents response with pooled sera of both lesioned groups.

noted inhibition of corpus luteum formation in the ovary(2-6,10,11). Uterine hypertrophy can occur however without change in ovarian weight(2-5) and small ovaries may exist with or without enlarged uteri(11,10). These inconsistencies probably reflect 2 experimental variables; namely, degree to which hypothalamic lesion damages the arcuate nucleus-median eminence complex, and duration of persistent estrous condition. The high proportion of estrous animals produced was well correlated with placement of lesions in an area limited anteriorly to the pre-optic region, terminating posteriorly just short of the anterior tip of median eminence. Inasmuch as severe damage to median eminence and the associated peri-ventricular grey area usually results in profound atrophy of the reproductive tract and obesity(1,12), it is conceivable that the spectrum of results obtained, ranging from persistent vaginal cornification through varying degrees of genital tract involvement, actually reflects gradations in quantitative rather than qualitative damage to components entering in or comprising the median eminence. With time, the hormonal balance in persistent estrous rats may change so that some atrophy of the ovary co-exists with hypertrophied uteri. Change in target organ sensitivity also cannot be precluded. The precariousness of the pituitary-ovarian hormonal balance in such rats is easily demonstrated by exposing them to cold (5°C; 12-52 days) whereupon both ovary and uterus involute and the persistent vaginal cornification is replaced by anestrous condition(13).

The existence of discrete FSH and LH centers in the hypothalamus regulating cyclicity and other phases of reproductive activity does not appear to gain support from this study. The results, however do support the interpretation given by Flerko(6,7) namely that anatomic integrity of the rostral hypothalamic area in question is necessary for normal estrogen-FSH interaction. Inhibition of compensatory ovarian hypertrophy in persistent estrous rats, considered "pari passu" with bioassay data, indicates that hypothalamic lesions interfered not only with cyclic release of LH(2-5,12) but with pituitary FSH function as well. It is evident that FSH secretion, though probably constant, is not proceeding at optimal levels. The gonads are not enlarged, as would be expected if FSH secretion were maximal; moreover, compensatory hypertrophy of the gonad, a phenomenon known to require augmented FSH secretion, is inhibited. The demonstration that the FSH blood level in unilaterally castrated lesioned rats is significantly lowered is consonant with this interpretation. It cannot be conclusively stated as to whether curtailment of FSH secretion involved synthesis as well as release inasmuch as hormonal stores in pituitaries of ovariectomized, lesioned rats were at least equal to those of control glands. Estimation of both FSH and LH content in pituitaries of persistent estrous rats with intact gonads would be of great interest in view of eventual pituitary hypertrophy.

Summary. Electrolytic lesions in pre-optic and anterior hypothalamic areas of the brain induced persistent (frequent) vaginal estrus and uterine hypertrophy in rats examined 25-300 days post-operatively. Gonad weight eventually decreased. Compensatory hyper-

trophy of ovary after unilateral castration was inhibited in persistent estrous lesioned rat. FSH stores in adenohypophysis were maintained but titers in serum decreased. The results signify that adenohypophysis of persistent estrous lesioned rat loses its capacity to augment secretion of FSH under conditions of enhanced demand (unilateral oophorectomy).

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Comparison of Cholesterol and Estrogen-Induced Atherosclerosis in Cockerels.* (25754)

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Atherosclerosis can be induced in cockerels by administering estrogen(1,2) and by feeding a diet high in cholesterol(3). A comparison of results obtained by these procedures, separately and combined, has been made.

Material and methods. Method used in this laboratory for studying quantitatively the atherogenic response of cockerels to exogenous estrogen has been described(4). Cholesterol induced atherosclerosis was produced in White Leghorn cockerels, 4-5 wks old, by feeding chick growing mash containing cholesterol deposited on the diet from ethyl ether solution. Atherosclerosis was evaluated as % area of the aorta, brachiocephalic, and iliac arteries involved in plaque formation. Other procedures were similar to those described previously(4).

Results. Cholesterol-induced atherosclerosis. Graded levels of cholesterol fed to cockerels for 8 wks resulted in progressively larger values for degree of atherosclerosis, serum cholesterol, and phospholipid (Table I). Feeding .5% cholesterol diet longer than 8 wks did not elevate these values (Table II). Increasing dietary cholesterol to 1% resulted in lower serum cholesterol and phospholipid values in 16 and 20 wks than either 8 or 12 wks. Degree of atherosclerosis reached a peak at 12 wks which was twice as great as either dietary cholesterol level in 8 wks. Although increasing the amount of dietary cholesterol to 1% resulted in elevated serum cholesterol and phospholipid values, sensitivity to atherosclerotic vascular involvement was decreased. These data indicate that feeding young cockerels a .5% cholesterol diet for 8 wks is optimal for inducing hypercholesterolemia and plaque formation in the major arteries. This method for producing cholesterol-induced atherosclerosis in cockerels has been adopted for studying the disease in this laboratory.

Cholesterol-estrogen induced atherosclerosis. Comparison of results obtained with cholesterol and estrogen as atherogenic agents, separately and combined, during 8 wks was made (Table III). Although serum cholesterol, phospholipid, and polysaccharides in-

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creased with larger doses of estrogen, % of aorta, brachiocephalics, and iliacs involved in plaque formation was greater at the 5 mg dose of estrogen than when smaller or larger amounts were injected. Feeding cholesterol diet to estrogen-treated birds augmented hy-

TABLE I. Dose Response of Cockerels to Dietary Cholesterol—8 Week Period.

Cholesterol added to diet (%)	No. cockerels	Atherosclerosis (% area)	Blood serum values: Cholesterol Total (mg %)	Cholesterol % free/total	Phospholipid (mg %)
.0 (controls)	24*	.03	113	27.1	225
.25	4	.9	186	27.2	151
.5	24*	17.9	391	28.3	249
1.0	12†	22.9	668	28.8	326
2.0	6	33.5	1189	27.8	406

* In 4 groups. † In 2 groups.

TABLE II.* Time and Dose Response of Cockerels to Dietary Cholesterol.

Exp. period (wk)	Cholesterol added to diet (%)	Atherosclerosis (% area)	Blood serum values: Cholesterol Total (mg %)	Cholesterol % free/total	Phospholipid (mg %)
8	.0	.1	121	26.1	190
8	.5	16.8	303	28.4	172
8	1.0	18.5	851	27.9	346
12	.0	1.6	99	28.2	169
"	.5	18.0	353	28.2	216
"	1.0	38.9	722	28.5	309
16	.0	.2	98	25.1	164
"	.5	19.4	243	25.8	148
"	1.0	28.9	330	23.5	179
20	.0	1.2	94	25.9	174
"	.5	11.4	312	24.9	213
"	1.0	25.7	379	24.8	229

* Each group consisted of 5 or 7 cockerels excepting 1 group of 4.

TABLE III. Dose Response of Cockerels to ECP, with and without Cholesterol Added to the Diet—8 Week Period.

ECP,* mg/bird (IM)	Cholesterol added to diet (%)	Atherosclerosis (% area)	Blood serum values: Cholesterol Total (mg %)	Cholesterol % free/total	Phospholipid (mg %)	Total polysaccharides (mg %)
Controls						
.0†	.0	.02	100	21.3	174	72
.0‡	.5	16.2	227	26.5	162	76
1.0	.0	.3	109	22.0	208	74
1.0	.5	12.0	273	30.8	214	81
5.0	.0	22.8	383	58.1	1206	117
5.0	.5	19.5	839	37.3	1243	120
7.5	.0	9.2	746	65.3	1986	163
7.5	.5	22.2	1280	42.2	1968	171
10.0	.0	10.2	995	78.1	4423	181
10.0	.5	24.0	1260	49.5	2954	161

* Reg. U. S. Pat. Off., Upjohn Co. brand of Estradiol Cyclopentylpropionate dissolved in cottonseed oil.

† Nine cockerels in 2 groups.

‡ Eleven cockerels in 2 groups. Other groups consisted of 5 or 6 cockerels.

Dosage equivalent administered to each bird on a 10-day basis.

TABLE IV.* Response of Cockerels to ECP and Dietary Cholesterol—1 Week Period.

ECP, mg/bird (IM)	Cholesterol added to diet (%)	Atherosclerosis (0 to 4+)	Blood serum values: Cholesterol—Total (mg %)	Blood serum values: Cholesterol—% free/total	Blood serum values: Lipoprotein, mg % cholesterol in: Alpha	Blood serum values: Lipoprotein, mg % cholesterol in: Beta
.00	.0	.0	166	24.5	84	100
.00	.5	.0	372	27.0	53	339
3.75	.0	2.2	343	31.9	11	368
3.75	.5	2.2	623	23.5	2	660

* Each group consisted of 5 or 6 cockerels.

percholesterolemia, but serum phospholipid and polysaccharide values were unchanged except in birds injected with 10 mg estrogen, when lower values were observed. Influence on plaque formation of feeding cholesterol to estrogen-treated birds resulted in relatively small increases beyond the 5 mg dosage.

Combining .5% dietary cholesterol with estrogen treatment at the 5 mg level over an 8 wks period provides another useful method for studying atherosclerosis in cockerels, since there is a progressive increment in plaque formation and hypercholesterolemia, as well as serum phospholipids and polysaccharides in the cholesterol fed birds between the 1 mg and 7.5 mg doses of estrogen, indicating that this is a sensitive area of response.

Using a modification of this method for a 1 wk experiment, birds were fed .5% cholesterol diet and injected with 3.75 mg of estrogen (Table IV). Under these conditions it is necessary to use an alternate method for evaluating atherosclerotic plaque formation (4). Although the cholesterol diet alone caused hypercholesterolemia and induced alpha and beta lipoprotein patterns characteristic of atherosclerosis, no plaques were formed during the 1 wk period. This is in contrast to the 8 wk experiment described above in which plaques were associated with similar serum cholesterol values. It is of considerable interest also that cockerels injected with estrogen and those fed cholesterol diet had comparable hypercholesterolemia, but plaques were found only in the hormone-treated birds. Combining dietary cholesterol with estrogen treatment resulted in 2-fold increases in serum cholesterol and beta lipoprotein; however, the degree of plaque formation was not altered.

Summary. Method is described for inducing atherosclerosis in cockerels by feeding chick growing mash containing .5% commercial cholesterol. Procedures for inducing atherosclerosis in birds by exogenous estrogen have been published(4). Experimental procedures are presented also for studying atherosclerosis induced in cockerels by combining estrogen and cholesterol treatment. Comparison of artery and blood serum changes associated with this disease as induced by these methods shows that a critical level of estrogen causes more rapid plaque formation than cholesterol, and that degree of hypercholesterolemia does not determine extent of atheromatosis.

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Modification of Grollman's Method for Induction of Renal Hypertension.* (25755)

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Grollman's method for producing hypertension in rats is probably the simplest and most convenient procedure available(1). As is well known, this method consists of uninephrectomy and constriction of the remaining kidney by a figure-of-eight ligature. However, the yield of hypertensive rats is relatively small. In our hands, about 15 to 20% of the rats are found hypertensive 4 to 5 weeks following this operation. Efforts to increase the yield by a moderate increase in tension of the ligature resulted in a mortality of about 80% within 3 days, compared to about 40% usually encountered with the routine procedure after this interval. The cause of death following renal ligation was suspected to be uremia. Thus, the increase in intra-renal tissue pressure from ligature compression, combined with the swelling from manipulation of the kidney, could result in anuria by obliteration of the tubules. It was surmised that if such an increased intra-renal pressure could be reduced, many potentially hypertensive rats might survive the operative effects and become useful experimental animals. It seemed worthwhile to administer the anti-inflammatory steroid, prednisolone, postoperatively, in an effort to suppress the suspected fatal renal swelling in order to increase the yield of hypertensive rats.

Method. One hundred Long-Evans male rats, 4 to 5 weeks old, were subjected to Grollman's procedure as usually done in this laboratory. Another group of 100 similar rats were similarly operated upon, but in addition were injected subcutaneously with 5 mg prednisolone† in aqueous suspension. The animals of both groups were observed for fatalities for 5 to 5 weeks. Blood pressure determinations were made at this time(2), and those rats whose blood pressures were over 140 mm Hg systolic were classified as hypertensives.

Results. Table I presents results. The data obtained show that administration of prednisolone, postoperatively, reduces immediate mortality from 42% to 11%. In conjunction with improvement in survival rate, yield of hypertensive rats 4 to 5 weeks later was more than doubled. Apparently many of the rats which ordinarily succumb to renal ligation were potentially hypertensive. Prednisolone treatment protected many of these animals from the immediate effects of operation, resulting in a salvage of significant numbers of hypertensive rats.

It is readily acknowledged that the mechanism by which prednisolone prevented death of many of the rats following renal ligation has not been demonstrated. Direct evidence was not obtained to indicate that the anti-inflammatory steroid prevented death from anuria by reducing the suspected renal swelling. On the basis that this steroid produced results which conformed to its well-known physiological activity, such a deduction is not unreasonable. Regardless of the actual mechanism, this modification of Grollman's procedure appears to be effective in increasing yield of hypertensive rats.

These results provoke the thought that in certain cases of anuria encountered clinically, where increased intra-renal pressure is suspected to be a major factor, some benefit might be obtained from corticosteroid therapy.

Summary. Administration of prednisolone

TABLE I. Effect of Prednisolone on Induction of Hypertension by Renal Ligation.

Group	No. of rats	72-hr mortality	Subsequent mortality	No. of non-hypertensive	No. of hypertensive
Untreated	100	42	10	32	16
Treated with prednisolone	100	11	16	36	37

* Aided by grant from Nat. Inst. Health, Nat. Heart Inst.

† Prednisolone was supplied through courtesy of Schering Corp., Bloomfield, N. J.

to rats subjected to Grollman's method for inducing renal hypertension lowered immediate mortality rate. It was deduced that the steroid was effective in combating intra-renal pressure suspected to be a frequent cause of death, resulting in a higher yield of hypertensive rats.

Addendum. According to Grollman, there is a low mortality and a high yield of hypertensive rats in a two-stage operation, the renal ligation first, and several weeks later the nephrectomy. The above report is significant, therefore, only when a one-stage operation is desired.

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Fluorescence of Tetracyclines in Filarial Worms.* (25756)

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Antibiotics of the tetracycline series fluoresce a yellow-gold color when stimulated with long wavelength ultraviolet light. Following administration of these antibiotics to animals, a diffuse, induced fluorescence has been observed throughout soft tissues with no special affinity for these tissues(1,2). However, studies have shown (3,4) that these drugs localize *in vivo* in newly proliferated normal bone tissue or new bone formations associated with certain neoplasms (unpublished). Recently, we have demonstrated that tetracyclines are deposited in filarial worms *in vitro* following exposure to drug and that yellow fluorescence of worms is sufficiently pronounced to permit their visualization *in vivo* following administration of drug to human or animal hosts. The following report describes these studies with special emphasis on fluorescent detection of worms in subcutaneous lesions of a patient with filariasis.

Materials and methods. Patient study. The patient was a 15-year-old white female who had spent most of her life in French Equatorial Africa, an area endemic for infection with the "eye worm," *Loa loa*. On admission, she had "fugitive swellings" on trunk and extremities and throughout her hospital course there was often seen in the center of these swellings a white, elevated band which moved about and appeared to be a subcutaneous migrating worm. On one occasion by application of heat to the forearm, a migrating worm was made to appear subcutaneously, then made to disappear by application of ethyl chloride spray. On 6 occasions, both night and day, blood samples from the patient were examined by Knott's technic(5) and in one daytime specimen the unsheathed microfilariae of *Acanthocheilonema perstans* were demonstrated. Although sheathed microfilariae were not detected, it is likely that the patient also had *Loa loa* infection since, in Africa, infection with *A. perstans* is frequently associated in patients with loaiasis(6). The patient proved markedly sensitive to dog heartworm (*Dirofilaria immitis*) antigen, giving a 3+ intradermal reaction to a 1:48,000 dilution of antigen. Tetracycline, in a dosage of 1 g daily by mouth, was given to the patient. At intervals thereafter, the patient's skin was fluoresced with a 9-watt hand lamp emitting ultraviolet at 3660 Å. *In vitro experiment with D. immitis.* Approximately 0.25 ml of heparinized dog blood containing living microfilariae of the dog heart-worm was pipetted into 2.5 ml of a buffered solution of chlortetracycline containing 1 mg/ml. After 5 minutes exposure to the antibiotic, a sample was taken and a slide prepared. At the end of 45 minutes exposure, remaining microfilariae in the drug solution were washed by centrifugation 2 times with buffered saline, pH 7.0, and a slide prepared from the sediment

* The authors thank Charles B. Evans for valuable technical assistance.

after the last centrifugation. Both 5 and 45 minute wet-mount preparations were examined at fluorescence microscopy using a Leitz Ortholux microscope equipped with a 1000-watt General Electric AH-6 mercury arc lamp(7). Using a Corning No. 5840 transmitting filter (2.25 mm thick), living microfilariae were stimulated with ultraviolet light at a wavelength of 3650 Å. A Wratten 2A ultraviolet absorbing filter was used in the eyepiece of the microscope. *In vivo experiment with D. immitis.* A 25 kilo dog infected with *D. immitis* was given a single I.V. injection of tetracycline in a dosage of 40 mg/kilo. Samples of heparinized blood taken ½, 1½, 3, 24, and 96 hours later were examined at fluorescence microscopy to determine uptake of drug by microfilariae. At a later date, the same dog was given the same I.V. dose of tetracycline. The animal was killed 30 minutes after cessation of injection, the adult *D. immitis* worms recovered from the heart, and grossly fluoresced with a 9-watt ultraviolet hand lamp. Subsequently, the worms were quick-frozen in petroleum ether at −65°C(8), fresh-frozen sections cut in a cryostat, and the sections mounted in a medium containing 9 parts of glycerine and 1 part of buffered saline, pH 7.0.

Results. Patient study. Twenty-four hours after initial dose of tetracycline, the patient was placed in a dark room and the skin fluoresced with a UV hand lamp. Linear, yellow-fluorescent streaks were seen on many parts of the body. When traced at intervals over a 6-hour period, streaks were seen to migrate in a circular fashion over a 5 to 6-inch area. A photograph of the patient's forearm, taken under ultraviolet light, localized the subcutaneous worms as yellow-fluorescent tracts (Fig. 1). From morning to afternoon or to the following day, the fluorescent tracts changed in location. For example, one day a fluorescent tract was observed behind the patient's left ear and the following day behind the right ear. This may have represented the same worm in its subcutaneous migrations. From these observations, it appeared evident that the worms had selectively taken up a considerably larger amount of drug than the surrounding host tissues since

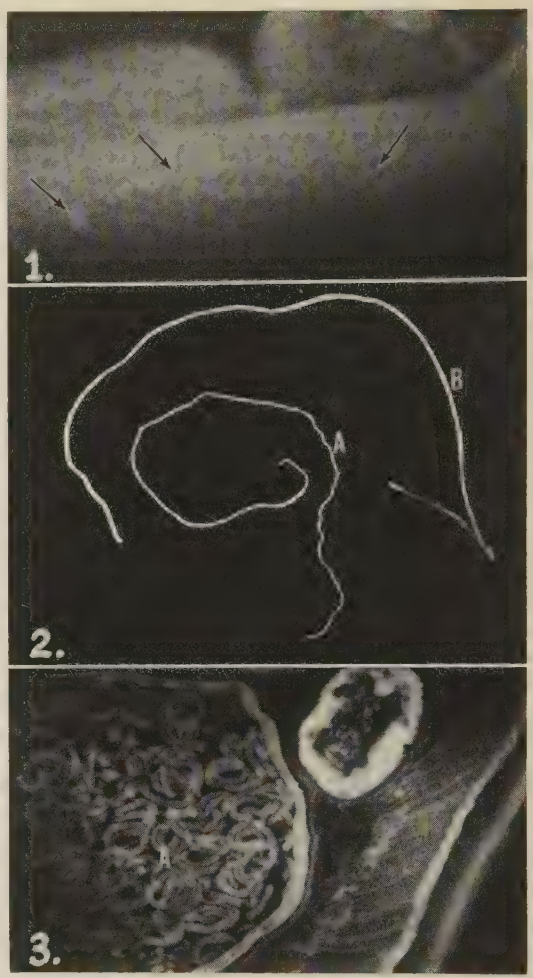

FIG. 1. Photograph of patient's forearm under ultraviolet light showing 3 fluorescent tracts (arrows).

FIG. 2. Fluorescence photograph of adult *Dirofilaria immitis* worms after *in vivo* exposure to tetracycline. A. Male. B. Female.

FIG. 3. Fluorescence photomicrograph of fresh-frozen cross section of adult female *Dirofilaria immitis* after exposure to tetracycline. A. Fluorescence of microfilariae *in utero.* × 172.

only discrete tracts on the skin showed induced yellow fluorescence in contrast to blue-gray autofluorescence of uninvolved skin areas. At times during tetracycline therapy, no fluorescent tracts were observed on the skin. This was probably a function of the depth of the worms in subcutaneous tissue and, therefore, lack of ultraviolet stimulation.

In vitro experiment with D. immitis. The microfilariae of *D. immitis* which had been exposed to a solution of chlortetracycline for

5 minutes were examined with a fluorescence microscope. They fluoresced a yellow color at approximately a 3+ intensity. In each embryo, the drug had been deposited in the central column of nuclei so the internal structure of microfilaria was clearly visible. During examinations, yellow fluorescence of microfilariae showed rapid fatiguing of an irreversible nature, a process frequently due to a photochemical reaction accompanied by oxidation(8). The embryos exposed to antibiotic for 45 minutes, then washed, fluoresced brilliantly (4+) but the yellow color fatigued at the same rate as in those exposed for 5 minutes. Microfilariae not exposed to drug exhibited blue-gray autofluorescence. Living microfilariae which first had been exposed to chlortetracycline, then stimulated with UV light, were killed in about 1 minute. However, without drug exposure, it took about 10 minutes for them to die.

In vivo experiment with D. immitis. To obtain more direct evidence of uptake of tetracycline by adults and embryos of a filarial worm, *in vivo* studies were conducted on a dog infected with dog heartworm. One-half hour and 1½ hours after I.V. administration of tetracycline, blood samples were taken and examined at fluorescence microscopy. As in the case of the *in vitro* studies, the central column of nuclei in each microfilaria obtained from the dog fluoresced a bright yellow color. In blood drawn 3 and 24 hours after drug was given, the microfilariae still contained drug but in somewhat smaller amounts, as shown by their being slightly less fluorescent. Microfilariae examined 96 hours after tetracycline administration showed no fluorescence. Fluorescence of the parasites observed in the ½, 1½, 3, and 24 hour samples fatigued quite rapidly under ultraviolet stimulation and the light was lethal to them in about 1 minute.

To demonstrate that tetracycline was specifically deposited in adult worms, the dog was given a single I.V. injection at a later date. The adult worms recovered from the heart and fluoresced with a UV hand lamp were a brilliant yellow-gold color, demonstrating presence of considerable amounts of drug. Some of the worms were quick-frozen and after 4 months were still brilliantly fluorescent (Fig. 2). Microscopically, in frozen sections of the adult worms, the drug was localized by its yellow fluorescence throughout most of the internal structures (Fig. 3). Of particular interest was the observation that the drug had been deposited in the uterus of the female as well as in the embryos contained within the uterus. Cuticula of the worms contained no drug, as was evidenced by a blue autofluorescence. Adult specimens of *D. immitis* not exposed to tetracycline exhibited a blue autofluorescence.

The selective concentration of tetracyclines by microfilariae and adult worms in host tissues has at least 2 therapeutic implications. First, tetracyclines themselves may have some antifilarial effect since treated microfilariae survived less well under ultraviolet stimulation than did untreated specimens. Second, if it were possible to combine some active antifilarial compound with tetracycline, it might permit a greater and more rapid concentration of antifilarial drug in worms within the tissues of the host. Since the tetracyclines can be localized and visualized in worms by fluorescent methods, this might serve as an effective tool in study of mode of action of these drugs. The observation that filarial worms can be visualized by a simple fluorescent method in a human subject after administration of tetracycline offers certain diagnostic possibilities.

Summary. 1. After administration of tetracycline to a patient having migrating, subcutaneous filarial worms, parasites were localized and visualized as yellow-fluorescent tracts beneath the skin following stimulation with UV light. 2. Exposure of microfilariae of *D. immitis in vitro* to a solution of chlortetracycline resulted in uptake of antibiotic by embryos in amounts which were readily visible by fluorescent methods. 3. *In vivo* studies on a dog infected with *D. immitis* revealed that the central column of nuclei of each microfilaria specifically took up tetracycline. The drug also was deposited in adult worms making them brilliantly fluorescent. In fresh-frozen sections of an adult female worm, the drug was localized throughout internal structures including uterus with its contained embryos.

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Congenital Malformations in Offspring of Mice Treated with Caffeine.* (25757)

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Certain substances which act as mitotic poisons have been reported to have teratogenic effects in mammalian embryos(1,2). However, many chemicals present in foods, beverages or drugs have not been tested or have been insufficiently studied for possible teratogenic effects. A few studies on caffeine have been reported. Druckrey and Schreiber(3) observed cessation or irregularity in mitosis in fertilized eggs of sea urchin after these were dipped in a caffeine solution. Eichler and Mügge(4) found no deleterious effects on embryonic development when pregnant rats were injected subcutaneously with large doses of caffeine. However, continuous treatment of pregnant rabbits with high doses of caffeine resulted in occasional early embryonic death or retarded development; the newborn young appeared to have "poor resistance"(5-7). Since no congenital malformations have been reported, we have investigated the effects of high doses of caffeine injected in pregnant mice.

Methods. Approximately 100 pregnant mice of the SMA strain (originating from Japanese Nat. Inst. of Genetics) were given a single intraperitoneal injection of 1% caffeine once during 7th to 15th days of pregnancy. A dosage level of 0.25 mg/g body weight, considered to be approximately the maximum dose tolerated, was used. The animals were sacrificed near term or in midpregnancy and fetuses weighed, examined for anomalies, and fixed in 20% formalin solution. Fetuses obtained from 25 normal pregnant mice were used as controls.

Results. Table I shows that caffeine administration may result in embryonic death or in malformed fetuses. Incidence of embryonic death was highest for injections given during 7th-12th days, when resorption of all embryos of a litter was sometimes induced. However, any injection given between 7th-14th days resulted in some dead or macerated fetuses. Malformations were observed in 18 to 43% of fetuses when injections were given between 10th and 14th days; a single malformation was found for injections on 9th day

TABLE I. Effects of Intraperitoneal Injection of Caffeine* in Pregnant Mice Observed at Term.

Day of inj.	No. of mice	No. of complete resorptions	Young		
			Total No.	% dead	% abnormal
7	4	2	11	18	0
8	5	0	36	6	0
9	11	2	45	11	2
10	6	0	40	3	38
11	10	0	57	12	19
12	8	1	41	17	41
13	7	0	51	8	43
14	7	1	44	18	18
15	3	0	24	0	0
Uninjected controls	25	0	225	2	0

* 1% aqueous solution, 0.25 mg/g body wt.

* We are indebted to Dr. M. M. Nelson, Dept. of Anatomy, University of California, San Francisco, for advice and aid in preparation of manuscript.

TABLE II. Number and Types of Malformations Observed in Offspring of Pregnant Mice Given Intraperitoneal Injections of Caffeine.*

Day of inj.	Abnormal young No.	No. of young with specific malformations in: Digits Fore	Hind	Joints†	Palate	Hematoma	Other
9	1				1		
10	15	3	10	3	2		1
11	11	8	8		1	1	
12	17	14	9	4	10	12	
13	22	14	7	2	9	15	1
14	8	1	8	2		4	

* 1% aqueous solution, 0.25 mg/g body wt.
† This column includes clubfoot as well as joint malformations.

of pregnancy. No significant effects on embryonic body weights were observed. All placentae were normal in size, shape, and color and histological study of representative placentae for each litter did not reveal any morphological changes.

The malformations observed were predominantly those of skeletal system, especially digital defects and cleft palate (Table II). Digital defects included angulation, brachydactylism, syndactylism, adactylism, and polydactylism. The critical period for adactylism in forefeet was 10th-13th days and in hindfeet 11th-14th days. Clubfoot (*pes varus*) and joint malformations were also observed. Cleft palate was induced by injections between 9th and 13th days with the highest incidence observed for injections given on 12th or 13th day. All cleft palates were bilateral and complete but of varying widths. One cleft palate was accompanied by harelip, in which a cleft of left premaxillary bone as well as corresponding upper lip was found. Cleft palates were often associated with hematomas in subcutaneous or deeper layers of upper and lower jaws. Adactylism and brachydactylism were also usually associated with superficial hematomas. In some cases these were comparatively small, localized hematomas accompanied by congestion of adjacent blood vessels in subcutaneous tissues. In other cases large hematomas were present in deeper tissues and were often associated with absence, hypoplasia or deformity of phalanges and metacarpal bones, and disturbances of muscle development.

Subcutaneous hematomas were observed when an injection was given on or after 11th day (Table II). When pregnant mice were sacrificed in mid-pregnancy, hematomas could be recognized as early as 12 hours after injection but were most frequent in older embryos of 15-16 days fetal age. In earlier stages hematomas were usually confined to fore and hind feet but in later stages they occurred in other areas such as maxilla, mandible and tip of tail. Number and size of hematomas increased with length of time following injection. Although there appears to be a correlation between hematomas and certain malformations, other defects observed in this study such as malformed elbow joints, *pes varus* and polydactylism were not accompanied by hematomas. The association of digital deformities with hematomas resulting from caffeine administration appears to be similar to results obtained with deficiencies of linoleic acid(8) and pantothenic acid(9,10).

The placental transfer of caffeine observed in rabbits and dogs(11) and the lack of macroscopic or microscopic changes observed in the placenta in this study permit the suggestion that caffeine may have directly affected the developing embryo.

Summary. Pregnant mice were given single intraperitoneal injections of caffeine on different days of gestation. When injections were administered between 9th and 14th days, malformations, predominantly in the skeletal system, were observed. Superficial hematomas observed in vicinity of anomalies may have been concerned in development of some of these malformations. No macroscopic or microscopic changes were observed in placentae of malformed young.

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Trachoma Viruses Isolated in United States.* 1. Growth in Embryonated Eggs. (25758)

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It is believed that more than 400 million people are infected with trachoma throughout the world. Diagnosis of this disease rests largely on clinical impression (conjunctivitis, with follicles particularly on upper tarsus, papillary hypertrophy, vascular invasion of cornea, pannus, scarring) and typical cytology of conjunctival scrapings with intracytoplasmic inclusion bodies. These inclusions, described over 50 years ago(1) consist of elementary and initial bodies in a matrix of glycogen. Because of the morphologic appearance of elementary bodies and their developmental stages trachoma virus was accepted as a member of the psittacosis-LGV group(2) although the virus had not been unequivocally grown in the laboratory. Tang *et al.*(3) succeeded for the first time in propagating serially trachoma viruses from patients in China, by inoculating streptomycin-treated conjunctival scrapings into the yolk sacs of embryonated eggs. Subsequently similar viruses were isolated from patients in West Africa(4), Saudi Arabia(5), and Israel(7). Trachoma is no longer a prevalent infection in the United States, except on certain Indian reservations in the Southwest. Sporadic, active cases, are occasionally seen elsewhere. We wish to report the first isolation of "trachoma viruses" from patients in the United States. To date 6 virus strains have been isolated from trachoma patients presenting typical pictures of a) acute initial adult infection, b) relapse in adult infection of 20 years' standing, and c) early infection in Apache Indian children. A preliminary report on the first of these virus strains has appeared(8).

Materials and methods. Patients: Clinical diagnoses of trachoma were made by ophthalmological examination of patients referred to us or encountered in systematic surveys of Indian reservations. *Specimens.* From active cases of trachoma conjunctival scrapings were collected in 1 ml broth-saline containing streptomycin sulfate 1-10 mg/ml and at times also polymyxin B sulfate 0.1 mg/ml. Most specimens were collected from single patients, but some field specimens were pooled scrapings from 2-6 active cases. Field specimens from Arizona were kept at –20°C to –70°C during shipment for varying periods before processing. Specimens collected in San Francisco were kept for 1 hour at 4°C before inoculation. *Embryonated eggs*: Specimens were mixed thoroughly, then 0.5 ml was inoculated into the yolk sac of 6-8-day-old embryonated eggs obtained from a commercial source. Eggs were incubated at 35°C and candled twice daily. Deaths within 48 hours of inoculation were discarded. Blind passage of surviving eggs by the yolk sac route was carried out at first between 7 and 11 days after inoculation, later regularly at 7-day in-

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TABLE I. Sources and Characteristics of "Trachoma Viruses" Isolated in U. S.

Strain	Patient's description: Age, yr	Disease	Inclusions present	Serum C-F titer to psittacosis antigen	Specimen collected	No. egg passages needed for isolation: Elementary bodies	Deaths	Max egg infectivity: Passage level	LD_{50}/ml
Bour	36	Acute florid trachoma	+++	1:64	San Francisco	2	3	10	6.4*
Asgh	29	Relapse of cicatricial trachoma, 20 yr duration	++	1:4	"	1	1	7	7.4
Apache #1	7	Early active trachoma	—	1:2	San Carlos, Ariz.	4	5	7	6.7
#2	7	*Idem*	—	ND	*Idem*	2	2	ND	ND
#3	4	"	—	"	"	3	3	"	"
#4	10	"	—	"	Bylas, Ariz.	6	6	"	"

* Negative log of dilution resulting in 50% death of eggs. ND = Not done.

terval. Harvested yolk sacs were weighed, shaken with glass beads for 1 minute and made into a 20% suspension with streptomycin-containing broth-saline. The egg LD_{50} of established strains was estimated by inoculation of groups of 6-12 eggs with 10-fold dilutions of yolk sac suspension, recording deaths which occurred between 3rd and 12th day of incubation, and checking presence of elementary bodies in stained smears of yolk sacs from dead eggs. The egg LD_{50} was calculated by the method of Reed and Muench(9). *Microscopy*: Smears of conjunctival scrapings from patients were stained by Giemsa's method and examined for cytological picture(2), virus particles, and inclusion bodies. Impression smears of yolk sacs were stained with Giemsa's and Macchiavello's methods to detect initial or elementary bodies at a magnification of 1800 ×.

Results. To date 41 specimens from clinical trachoma have been subjected to 3-6 egg passages and 6 strains of "trachoma viruses" have been isolated. Isolates and their sources are briefly characterized in Table I. From specimens collected in San Francisco virus was isolated in the first (fresh material) or second egg passage (frozen material) whereas from 2-6 passages were required for appearance of virus in specimens collected in Arizona and kept at –20 to –70°C for varying periods. The San Carlos Apache Indian Reservation was visited twice. In Oct. 1959 4 specimens were obtained from children with early active trachoma one of which yielded virus in 4th passage (Apache #1). In Jan. 1960 with improvements in collection and shipping procedures conjunctival scrapings from 7 children with similar disease yielded 3 isolates (in passage 2, 3, and 6). Interestingly none of these 11 children had demonstrable inclusions in their conjunctival scrapings, although in other respects the cytologic picture was typical of active trachoma.

Elementary bodies were often seen in yolk sac smears one passage prior to occurrence of deaths among inoculated eggs. Morphologically and tinctorially elementary bodies resembled those of other members of the psittacosis-LGV group. They stained bright red-purple with Macchiavello's method and purple-blue with Giemsa, and measured about 250 mμ in diameter. They occurred diffusely in impression smears of infected yolk sacs and were occasionally grouped in inclusion body-like arrangements. However true intracellular inclusions have not been observed in our egg passages. The density of elementary bodies in smears from later egg passages was very high so that each microscopic field contained hundreds or thousands.

Early egg passages of fresh isolates resulted in irregular deaths. In later passages all eggs receiving a suitable inoculum died between 5th and 9th days after inoculation. For preparation of high titer pools 6-8-day-old chick embryos were inoculated with yolk sac suspensions diluted so as to cause death of half the eggs in 6-7 days. At that point the yolk sacs of surviving embryos were harvested

and pooled for storage at –40°C. Titers of such pools (Table I) reached 6.3 to 7.4 egg LD_{50}/ml of undiluted yolk sac suspension. Pools of strain ASGH regularly had a titer 1-1.5 logs higher than pools of strain BOUR; the reason for this difference is unknown. Infective titers of established strains, and ability to isolate ANY "trachoma virus" depended to a remarkable degree on fluctuation in egg quality, as observed by others(4,6). Strain BOUR grew well from Dec. 1958 until early Aug. 1959. For the next 2 months we could not propagate any virus. In Oct. 1959 the ability of eggs to support virus growth miraculously resumed. Suspensions of uninoculated yolk sacs from "bad" eggs apparently inhibited the infectivity of "good" virus for monkey eyes and "good" eggs. Strains established in the yolk sac grew readily also in the allantoic cavity. Using harvested allantoic fluid as inoculum for 3 consecutive allantoic passages did not impair infectivity *via* yolk sac route. However, quantitative data on this are still incomplete.

Isolates prepared as antigens by exposure to flowing steam for one hour, fixed complement with antisera to psittacosis virus to full titer. Concentrated suspensions of elementary bodies were toxic to mice upon intravenous injection. Instillation of egg-grown isolates into *M. cynomolgus* monkeys resulted in follicular conjunctivitis with typical intracytoplasmic inclusions. Isolates of similar egg infectivity differed in their pathogenicity for monkeys. No other laboratory animals could be infected. Strain BOUR produced typical acute trachoma with inclusion bodies in a human volunteer. Attempts to propagate our isolates in cultures of established cell lines have thus far given inconclusive results. Details of these studies will be reported.

Discussion. In certain areas of the world where trachoma is widespread the disease is often confused with non-trachomatous follicular conjunctivitis and even bacterial conjunctivitis, especially under survey conditions. Thus the diagnosis of "trachoma" in those areas designates a clinical rather than an etiological entity(5). If the finding of typical intracytoplasmic inclusions (indicative of viruses of the psittacosis-LGV group) were made mandatory to establish the diagnosis a large majority of cases could not be classified as "trachoma." Ability to cultivate "trachoma viruses" from inclusion-negative cases now permits the etiologic diagnosis of trachoma in such patients. In addition cultivation of "trachoma viruses" from different locations or different types of disease may permit comparison of pathogenetic and antigenic characteristics of these strains(6). Our initial results indicate that virus isolation from trachoma patients in the United States is still far from easy and seems to depend on many ill-defined factors. Recovery of virus was not dependent upon presence of inclusion bodies in patient's conjunctival scrapings. None of the Apache children yielding virus had any inclusion bodies, whereas an adult with the largest number of inclusions that we have ever observed failed to yield virus. Handling of the specimen may be of great importance in view of the relative instability of the agent. Perhaps the largest factor in successful isolation of "trachoma viruses" is the fluctuating susceptibility of embryonated eggs. At times strong inhibitors are present which make virus isolation or propagation virtually impossible. These inhibitors do not appear to be antibiotics transmitted from feed. Their nature and action are under study.

Summary. From 6 active trachoma cases in the United States strains of "trachoma virus" have been isolated. Conjunctival scrapings suspended in broth-saline containing 1-10 μg/ml streptomycin were inoculated into yolk sac of embryonated eggs. From 1 to 6 blind passages were required for establishment of strains which subsequently reached titers of $10^{6.3}$-$10^{7.4}$ egg LD_{50}/ml. The essential nature of ill-defined egg "quality" for isolation of "trachoma viruses" is discussed. The isolated agents produced unequivocal eye infections in *M. cynomolgus* and in a human volunteer.

We are indebted for many helpful suggestions to Drs. S. Bell, E. Murray, and J. Snyder of Boston and for aid in screening Indian children to Dr. S. Singh. Dr. B. Eddie kindly performed serological tests and provided antisera.

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Levels of Eosinophils, Platelets, Leukocytes and 17-Hydroxycorticosteroids During Normal Menstrual Cycle.* (25759)

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Ovulation is associated with thermal, chemical, cytologic and histologic changes in the human and affects levels of certain formed elements of blood(1-3). Eosinopenia has been observed during ovulation and menstruation, and it has been suggested that physiological stress at these periods may increase levels of adrenocortical hormones causing eosinopenia(3,4). The present study was concerned with relationship of levels of plasma 17, 21-dehydroxy-20-keto-steroids (17-hydroxycorticosteroids (17-OHCS) with levels of eosinophils, platelets and leukocytes during menstrual cycle.

Materials and methods. Twelve healthy women with normal menstrual cycles and one oophorectomized control subject were investigated. Two women were allergic. Oral temperature records were kept. Eosinophil, platelet, leukocyte and differential counts and plasma 17-OHCS determinations were performed between 12 noon and 1:00 p.m., to avoid diurnal variations. Approximately 11 to 13 counts and steroid determinations were obtained on each woman during mid-cycle and menstruation, as shown for one patient in Fig. 1. Direct platelet counts were done by phase microscopy(5) and eosinophils were counted by the Wintrobe method(6). Estimations of 17-OHCS in plasma were made by micromethod of Riondel *et al.*(7). Since it was desirable to obtain minimal amounts of blood from each woman daily, duplicate steroid determinations were not feasible. However, duplicate steroid determinations were done on 10 other normal women. The standard deviation of differences in values of the 2 determinations was 3.1. On the basis of these results, there is 95% confidence that an individual observation is no further from the true value than 7 μg%.

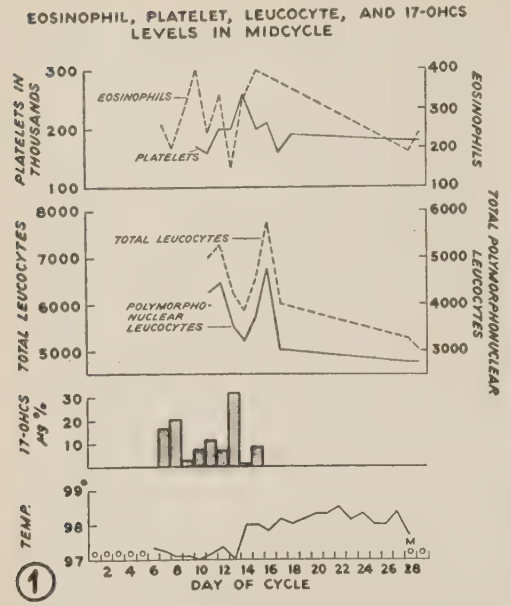
Results. A total of 183 eosinophil, platelet, total leukocyte and differential counts and 164 determinations of 17-OHCS levels were performed on 13 women with the results shown in Table I.

Eosinophil levels during menstrual cycle. Eosinopenia occurred during menstruation and on one day in mid-cycle in the majority of women. Menstrual eosinopenia occurred in 9 cycles (75%). However, the lowest eosinophil values of the entire cycle were usually in mid-cycle (Fig. 1). When basal

TABLE I. Results of Counts and 17-OHCS Determinations.

	Mean	Stand. error
Eosinophils	162.3	7.9
Platelets	254,955	3,907.–
W. B. C.	8,516	171.–
Polymorphonuclear leukocytes	5,483	267.–
Lymphocytes	2,573	56.–
17-OHCS	12	.71

* Supported by Sequoia Hosp. Auxiliary and Sequoia Hosp. Research Fund.



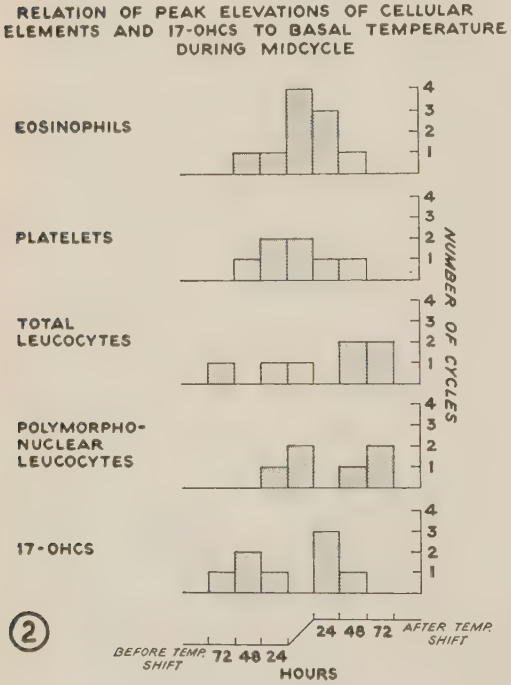


FIG. 1 and 2.

temperature graphs were correlated with eosinophil levels, eosinopenia occurred during ovulatory period in 10 cycles (83.3%) and was absent in 2 cycles (16.7%) (Fig. 2). The 2 allergic women had much more pronounced eosinopenia during mid-cycle than the non-allergic subjects.

Platelet levels during menstrual cycle. The platelets showed wave-like fluctuations during menstrual cycle with elevations in mid-cycle and depressions during menstruation in most cycles (Fig. 1). During menstruation, thrombocytopenia occurred in 8 cycles (66.6%). When basal temperature shifts were correlated with platelet levels, thrombocytosis developed during ovulatory period in 7 cycles (58.3%). In 5 cycles (41.7%) platelet elevations in mid-cycle were absent (Fig. 2).

Leukocyte levels during menstrual cycle. Leukocytosis occurred in mid-cycle in 7 cycles (58.3%). Polymorphonuclear leukocytes had mid-cycle elevations in 6 cycles (50%) (Fig. 1, 2). Levels of lymphocytes showed no significant variations during menstrual cycle. During menstruation, total leukocytes and neutrophilic leukocytes remained at normal levels in the majority of women.

17-OHCS levels during menstrual cycle. In each of 12 menstrual cycles, a peak value of 17-OHCS lasting 24 hours was noted in mid-cycle (Fig. 1). This represented highest steroid level in all but 3 cycles where highest values occurred during first and second day of menstruation. When 17-OHCS levels were correlated with temperature graphs, in 8 cycles (66.6%) the highest steroid levels occurred within 72 hours before temperature shift or within 48 hours after it (Fig. 2).

When steroid levels were correlated with the corresponding eosinophil values, in 6 cycles (50%) 17-OHCS were at peak values when eosinophils were at lowest levels. In 3 cycles (25%) the highest 17-OHCS levels occurred 24 hours before or after eosinopenia, in 2 cycles (16.7%) 48 hours before it, and in 1 cycle (8.3%) steroid peak occurred 72 hours before eosinopenia. Thus in all but one menstrual cycle steroid peaks occurred during or shortly before day of eosinopenia. During menstruation, 17-OHCS levels showed an inverse relationship to eosinophil levels in 6 out of 9 cycles (66.6%) in which steroid levels were determined. In the oophorectomized woman, a fairly close inverse relationship between steroid and eosinophil levels was established, but no significant variations in

eosinophil, platelet or leukocyte levels were found.

Comment. Since Pfeiffer and Hoff(8) first reported fluctuations of platelet levels during menstrual cycle, others have demonstrated thrombocytopenia during menstruation and thrombocytosis during the mid-cycle(2,9). When basal temperature graphs were correlated with platelet levels in the present study, it was again demonstrated that platelet elevations in mid-cycle occurred during the ovulatory period in the majority of women. It seems unlikely that thrombocytosis occurring in mid-cycle is related to increased 17-OHCS levels, since surgical stress is associated with thrombocytopenia(10).

Leukocytosis and increase of polymorphonuclear leukocytes in mid-cycle occurred in a majority of women. These may be attributed to a temporary increase of steroid levels, since other forms of stress produce leukocytosis (11).

Eosinopenia during the ovulatory period and during menstruation has been described, but explanations of the cause of this phenomenon have varied(3,4,12). The present study demonstrated eosinopenia in mid-cycle in 83% of women, even though eosinopenia and 17-OHCS peaks occurred on the same day in only 50% of women.

A diurnal inverse relationship between levels of eosinophils and 17-OHCS has been demonstrated previously in normal individuals(13). Although non-adrenal influences may affect the level of circulating eosinophils (14), it seems likely from our study that eosinopenia which occurred during ovulatory period in the majority of women resulted from increased 17-OHCS levels. In addition, menstrual eosinopenia found in a majority of women studied occurred in association with higher steroid levels. These data suggest that eosinopenia and increased 17-OHCS levels may result from stressful situations occurring during menstrual cycle.

Conclusions. Eosinopenia, thrombocytosis, leukocytosis, and increased 17-OHCS levels were demonstrated during ovulatory period. During menstruation, eosinopenia, thrombocytopenia and higher 17-OHCS levels occurred in the majority of women studied. It is suggested that stressful situations during ovulatory periods and menstruation may cause increased 17-OHCS levels with resulting eosinopenia.

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Radioautographic Analysis of Soluble Antigen-Antibody Complexes Separable by Paper Electrophoresis. (25760)

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The biological importance of soluble antigen-antibody complexes has been previously well established by inducing cutaneous reactions(1,2), anaphylaxis(2,3), and serum sickness(3,4), with soluble complexes prepared *in vitro*. The physical-chemical properties of such complexes have been studied by several technics including free boundary electrophoresis(5,6,7). Use of paper electrophoresis in analysis of soluble complexes, prepared for *in vivo* studies suggests itself, but its value is limited when conventional methods of staining are used in evaluating low concentrations of protein usually applied. In this study radioautographs of paper electrophoretic patterns containing radioactively labelled soluble complexes permitted a simple and sensitive method of analysis.

Materials and methods. Electrophoretically homogenous bovine serum albumin (BSA) was used for iodination and immunization procedures. Anti BSA sera were prepared by repeated injections of 250 mg of BSA mixed with Freund's adjuvant/injection for 6 week period. Fifty mg were injected into each of 5 portals, (intravenous, intramuscular, intraperitoneal, subcutaneous and intradermal), twice a week for 6 weeks. Rabbits were bled by cardiac puncture 5 days following last injection. Sera were pooled, frozen rapidly with alcohol and dry ice, then stored at −10°C. The γ globulin fraction was separated off as needed using ammonium sulphate method described by Cushing and Campbell(8). BSA was iodinated with one atom of iodine/molecule of protein according to the method of Talmage(9). Adequate specific activity was obtained by using .5 ml of solution containing 25 mg of BSA and 10 mc I^{131} with activity greater than 20 mc/ml, with .1 ml of .01 N KI carrier. The solution was dialyzed 2 days against water, then allowed to equilibrate with .86% NaCl. Free I^{131} was less than 2% on measurements of activity using 10% TCA protein precipitation. I^{131} γ activity was measured in a well-type detector coupled to conventional scaler. Background activity was 4.6 to 6 counts/second. Samples were counted for a sufficient time to keep statistical counting error below 1%.

Preparation and analysis of BSA-anti BSA complexes. BSA I^{131} was added to anti BSA γ globulin in proportions to give combination at point of equivalence. The mixture was allowed to stand at 27°C for 2 hours, then placed at 4°C for 24 hours. The precipitate was washed repeatedly and brought to a known volume with cold .86% NaCl. To insure adequate suspension of the precipitate, the mixture was agitated in a Ratheon sonic oscillator at 9 KC/sec for 100 seconds. Samples were set aside for future incubation with excess unlabelled antigen. Aliquots of samples were counted for radioactivity and nearly identical samples were selected for incubation with .5 cc of excess antigen at 2.5, 5, 10 and 20 times the equivalence zone. Insoluble antigen-antibody content represented by undissociated radioactive BSA corresponded to 82, 77, 73 and 69% respectively for 2.5, 5, 10 and 20 times the equivalence zone. Suspensions were incubated 6 hours at 4°C with constant agitation, then centrifuged repeatedly until activity in supernatants reached equilibrium. Similar .03 ml aliquots were run 20 hours at room temperature in Spinco tent type paper electrophoresis apparatus, model R - series D, using barbiturate buffer at pH 8.6, μ .075. After drying, radioautographs were prepared by tightly pressing Dupont medical 508 X-ray film against the papers for 48 hours. Film was developed in the usual way and autographs scanned in a Bender and Hobein densiometer. Paper electrophoretic patterns of soluble complexes and of control sera were similarly stained and evaluated. Optical density of each band in the radioautographs was expressed as percentage concentration as indi-

* Operated by Univ. of Chicago for Atomic Energy Comm.

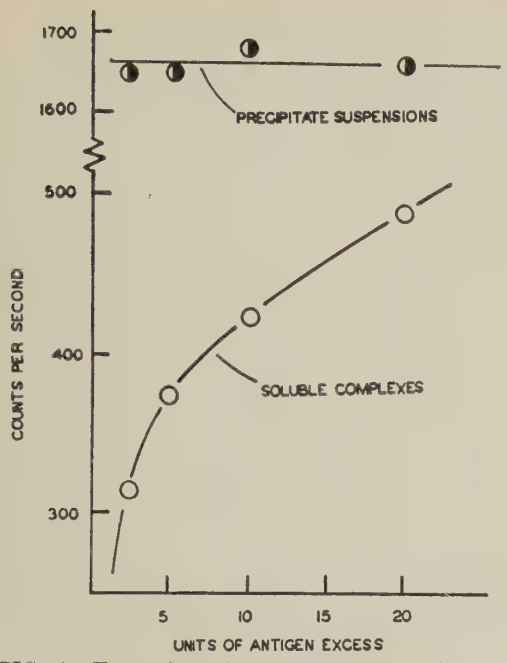

FIG. 1. Formation of soluble complexes is indicated by increasing radioactivity in supernatants with increasing amounts of antigen added in excess. At 20 × the equivalence zone approximately ⅓ of preformed labelled precipitate is dissolved in the form of soluble complexes, and aliquot measured contains 1 γ of BSA.

cated by the area under relevant parts of the tracing. Comparison with migration rates of normal rabbit serum proteins served to indicate relative electrophoretic mobilities of the soluble complexes. Control radioautographs of radioactive, electrophoretically homogenous BSA demonstrated 99% of isotopic activity located in albumin fraction.

Results. Micro-Kjeldahl analysis of labelled BSA indicated a specific activity of 470 counts/second for 1 γ of BSA I^{131}. Serial dilutions of labelled BSA, spotted on paper and radioautographed, revealed that concentrations of less than .1 γ of BSA were readily demonstrable in 48 hours. Aliquots of each supernatant, ranging in activity from 20-30 times this value, *i.e.*, containing 2-3 γ of soluble complexed BSA, dissolved in increasing antigen excess, were chosen for analysis (Fig. 1).

Radioautographs of the soluble complexes revealed 4 distinct bands (Fig. 2). Comparison of tracings of the autographs with tracings of control BSA and rabbit serum proteins demonstrated that the leading component migrated at the rate of normal BSA and that the remaining bands represented soluble complexes–one remaining at application point, and 2 migrating at the rates of β and α globulins respectively (Fig. 3). In previous work by Weigle *et al.* using BSA-rabbit anti BSA system, conventional methods of paper electrophoretic analysis revealed 3 bands. A band representing free BSA, and 2 bands migrating at rates of β and γ globulins were reported (10).

The most rapidly migrating complex is believed to be antigen rich on the basis of its migration rate and tendency of this fraction to increase in percentage concentration at the expense of the complex remaining at the application point, assumed to be antigen poor or antibody rich. The intervening band represents a complex of intermediate composition (Fig. 4). It is likely that the first 2 complexes represent the Ag2-Ab1 and Ag3-Ab2 complexes described by Singer(5,6,7), and that the varying weights and charges of the molecule account for their specific rates of electrophoretic migration. The fraction remaining at application point may be due to

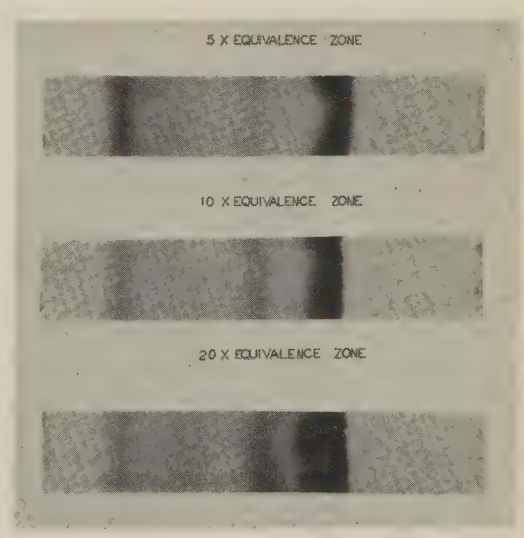

FIG. 2. Radioautographs of electrophoretic patterns of antigen-antibody complexes showing 3 bands: One remaining at application point, the other 2 migrating at rates of β and α serum globulins. Free BSA represented by the most rapidly moving fraction is almost completely absent in regions of low antigen excess with extensive washing of initial precipitate, but an excess is shown here for orientation.

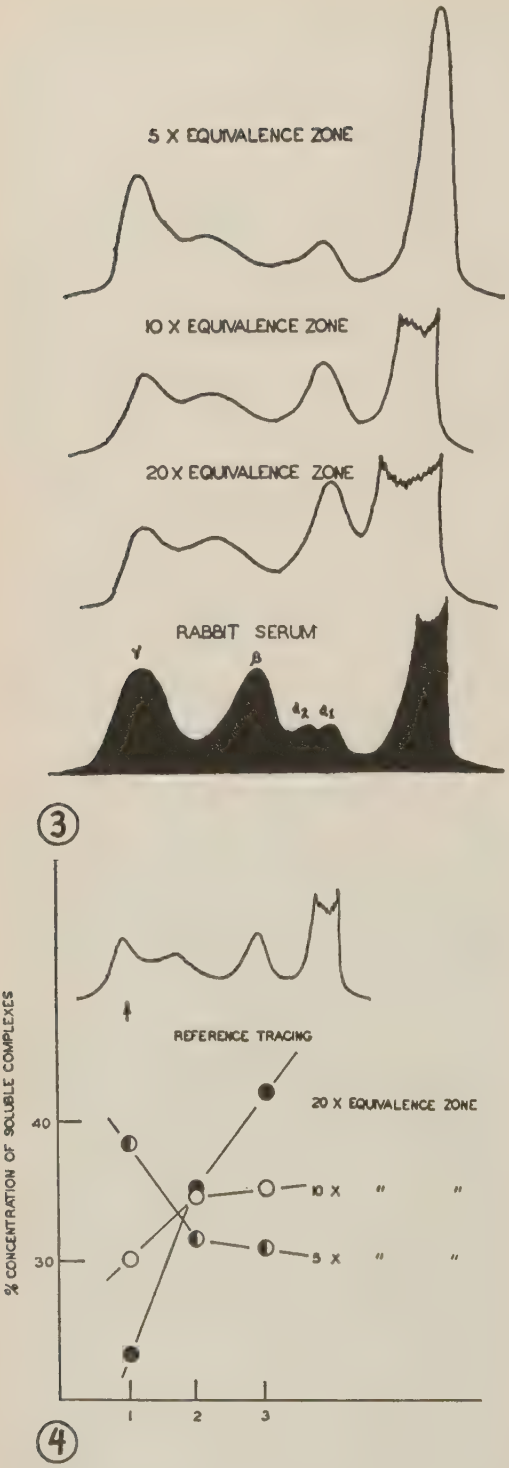

FIG. 3. Tracings of radioautographs of labeled soluble antigen-antibody complexes at 5, 10, and 20 × equivalence zone indicating their electrophoretic mobilities relative to a tracing of a stained paper electrophoretic pattern of control rabbit serum globulins. Leading fractions represent soluble complexes migrating at rate of α and β globulins, and the component remaining at application point may be reprecipitated complexes, or higher molecular weight complexes with a high affinity for the paper.

FIG. 4. Percentage concentration of each of the complexes at 5, 10, and 20 times the equivalence zone is indicated under the reference electrophoretic pattern for each of these complexes. The antigen-rich, fast migrating, complex increases in concentration at expense of the antigen-poor complex remaining at point of application. It would appear that the binding of BSA is divided equally between the 3 complexes in this system at a point somewhere between 5 and 10 times the equivalence zone.

a reprecipitation of insoluble aggregates as the system reequilibrated during time required for electrophoretic separation. Or the band may represent a soluble but higher molecular weight complex possessing a high affinity for paper supporting medium.

Summary. A radioautographic technic with a sensitivity many times greater than conventional staining methods is described for evaluation of paper electrophoretic studies of soluble antigen-antibody complexes. Electrophoretic mobilities of BSA-anti BSA soluble complexes relative to rabbit serum proteins are reported with a semi-quantitative analysis of percentage concentration for each of the complexes at various values of antigen excess.

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Virulence and Coagulases of *Staphylococcus aureus.** (25761)

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The ability of *Staphylococcus aureus* to produce coagulase has customarily been taken as an *in vitro* reaction indicating potential virulence for this organism. Evidence for the existence of 2 different kinds of coagulase was first reported by Duthie(1). The soluble coagulase causes clotting of plasma (fibrinogen) in presence of an accessory factor, whereas the bound coagulase causes clumping of the organism by plasma or fibrinogen alone. It is of interest to determine if either kind of coagulase is of itself an essential virulence factor. This was done by selecting a strain of *S. aureus* known to be virulent for rabbits and possessing both kinds of coagulase. Mutants lacking either kind of coagulase were then derived from this strain and checked for virulence in rabbits. A simultaneous loss of virulence could possibly indicate a direct relationship with the particular missing coagulase, whereas if the mutant retained its virulence in the absence of a coagulase then this factor could not be essential for virulence. The parent strain, *S. aureus* 18Z, used in these experiments, was chosen because it possessed several possible virulence factors, including the soluble and bound coagulases, alpha and delta hemolysins, leucocidin activity, hyaluronidase and fibrinolysin. It was sensitive to penicillin, streptomycin, and other antibiotics and was of the phage type 42 B/52/80/81. This strain was originally isolated from the human nasopharynx and was virulent for rabbits.

Materials and methods. To isolate coagulase negative mutants, 6 hour old cultures of *S. aureus* strain 18Z grown in trypticase soy broth were washed with saline and subjected to UV irradiation sufficient to reduce the population to 1% survival. The irradiated suspensions were then diluted and spread on trypticase soy agar containing 15% human plasma. On this medium coagulase positive staphylococci have been reported(2,3) to produce an opalescent halo around the colonies, presumably due to deposition of fibrin in the agar. Attempts to isolate coagulase-lacking mutants were made by picking colonies that formed either no halos or halos of much lighter density. The colonies thus selected were individually checked for production of the coagulases by the slide and tube tests. In testing for bound coagulase, 0.1 ml of an overnight broth culture, concentrated 5-fold by centrifugation, was mixed with 0.05 ml of human plasma on a depression slide and the cells observed for evidence of clumping for at least 5 minutes. In the case of bound coagulase-positive cultures, the clumping was usually seen within 1 to 3 minutes. Production of soluble coagulase was detected by mixing in a Wassermann tube 0.1 ml of concentrated culture, 0.5 ml of human plasma, and 0.1 ml of trypticase soy broth. The tubes were incubated in a water bath for 3 hours at 37°C and periodically examined for signs of clotting. This usually became evident within 1 to 2 hours in the case of soluble coagulase-positive organisms. Mutants which appeared to lack one kind of coagulase by these tests were rechecked several times using different lots of plasma from humans and normal rabbits to minimize the chance of observing negative coagulase reactions due to inhibition by antibodies which might be present in a particular sample of plasma. Mutants were tested for virulence by injecting normal rabbits either intracutaneously or intravenously with 10^8 organisms. Animals inoculated intracutaneously were observed for several days to see if local lesions developed. Rabbits inoculated intravenously were held for 7 to 10 days and then sacrificed. Organs of these animals were then examined grossly for staphylococcal lesions. In addition, samples of liver and kidney were plated out on trypticase soy agar to determine presence of viable organisms. In some experiments the staphylococci recovered from these organs were reexamined

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to note whether their coagulase activities were the same as for the organisms originally inoculated into the animal. Various activities of the strains were determined as follows: hemolysin patterns according to the method of Elek and Levy(4), leucocidin by the method of Valentine(5), fibrinolysin by the procedure used by Lack and Wailling(6), and hyaluronidase by means of the ACRA test(7).

Results. The screening method used to isolate coagulase-negative mutants by means of the plasma-agar medium did not prove completely satisfactory. At least 53 "haloless" colonies had been picked during a number of experiments, but were later found to produce both bound and soluble coagulase. Only one such "haloless" isolate proved to be a soluble coagulase-negative mutant. However, a few randomly picked "halo-forming" colonies did lack either one or the other kind of coagulase. From among all these isolates 3 were chosen and tested for virulence in rabbits. 1) Mutant 18Z-B, a halo producer like the parent 18Z strain, produced soluble coagulase, but lacked bound coagulase. 2) Mutant 18Z-C did not form halos on the plasma-agar medium. It was negative for soluble coagulase, but possessed the same amount of bound coagulase as the parent 18Z strain. 3) Mutant 18Z-D was originally isolated as a "haloless" colony, but on subsequent rechecking always produced a halo on plasma agar. It produced both kinds of coagulase. These 3 mutants produced the same reaction, insofar as halo formation is concerned, when grown on trypticase soy agar containing fibrinogen (fraction I) as when grown on plasma-agar. To minimize the possibility that the 3 isolates were not mutants of the parent strain, but contaminating staphylococci, the other characteristics of these isolates were compared with those of the parent strain. With the exception of 18Z-C which lacked fibrinolysin in addition to soluble coagulase all mutants exhibited the same characteristics† as the parent strain.

The results obtained in rabbits inoculated with the mutants were consistent. Rabbits given 18Z-B (lacking bound coagulase) or 18Z-C (lacking soluble coagulase) intradermally developed localized abscesses of the same type and severity as those produced by a comparable dose of the parent 18Z strain. When these 2 mutants were administered by the intravenous route, renal and, usually also, liver abscesses were found. The mutants could be isolated in large numbers from these organs and subsequent testing revealed that coagulase activity of the staphylococci recovered from these organs was identical with that of the mutant originally inoculated into the animal. Mutant 18Z-D (possessing both kinds of coagulase) failed to produce lesions by the intradermal route and could not be recovered from kidneys, or livers, of animals inoculated intravenously 7-10 days previously.

Since mutant 18Z-D gave every indication of being avirulent for rabbits, a comparison of the characteristics of this mutant was made with those of the parent 18Z strain to see whether any "virulence factor" had been lost which might account for the observed loss in virulence. The results demonstrated that the avirulent 18Z-D mutant was indistinguishable from the parent virulent 18Z strain in every property thus far tested.

Discussion. These studies indicate that *S. aureus* mutants lacking either bound or soluble coagulase are just as virulent for rabbits as is the parent strain possessing both kinds of coagulase. Therefore, the coagulases do not appear to be essential for virulence of this organism. This is not to say that the coagulases, either singly or in combination with other factors, play no role whatsoever in pathogenesis of staphylococcal disease since our data cannot shed light on this question. Furthermore, the data do not negate the value of testing staphylococci for coagulase production since these are substances commonly produced by the species in question. In fact the rather high correlation of coagulase production, and for that matter, alpha hemolysin and hyaluronidase production with observed virulence of *S. aureus* may result from the fact that these are all common properties of this species and that most members of the species are virulent. Yet the mere presence of these characteristics would not necessarily imply

† Alpha and delta hemolysins, hyaluronidase, fibrinolysin, leucocidin, phage type, lactose and mannitol fermentation, and sensitivity to 13 antibiotics.

that they are responsible for virulence.

The 18Z-C mutant actually appears to be a double mutant since it lost ability to produce fibrinolysin in addition to losing ability to produce soluble coagulase. Since this mutant is fully virulent it would appear that fibrinolysin is also not essential for virulence.

Isolation of the avirulent 18Z-D mutant likewise offered the possibility of identifying a factor which if lost results in loss of virulence. However, this mutant is identical with the parent strain in all aspects thus far studied with the exception of virulence. Further studies comparing these 2 strains are in progress.

Summary. Three mutants were isolated from a single strain of *Staphylococcus aureus* of known virulence for rabbits. Two mutants, one lacking the soluble type of coagulase and the other lacking the bound type of coagulase, were still virulent for rabbits. The third mutant which produced both kinds of coagulase had lost its virulence for rabbits.

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Correlation of Tryptamine-Induced Convulsions in Rats with Brain Tryptamine Concentration. (25762)

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Tedeschi *et al.*(1) recently reported appearance of clonic convulsions of forepaws of rats following i.v. injection of tryptamine (TA). A good dose response relationship was obtained between quantity of TA injected and relative frequency and intensity of resulting convulsions. This correlation suggested that the observed pharmacologic responses were associated with increased levels of TA in the brain. On the assumption that amine in rat brain was metabolized chiefly by monoamine oxidase (MAO), Tedeschi *et al.* postulated that inhibition of enzyme activity should prolong the elevated brain TA levels and, therefore, potentiate convulsant properties of the amine. Although pharmacologic expectations were fulfilled and procedure shown to be suitable for screening and pharmacologic evaluation of MAO inhibitors, the question of relationship with brain biochemistry remained unanswered. The purpose of our study was to determine whether the TA-induced clonic convulsions in rats pretreated with a MAO inhibitor were correlated with concentration of amine in the brain.

Procedure and methods. Male albino rats, Wistar strain, weighing between 175 and 225 g were used. Tryptamine hydrochloride in physiologic saline was injected into tail vein in dose of 5 mg/kg, calculated as free base. Trans 2-phenylcyclopropylamine hydrochloride (tranylcypromine)(2) and 1-isonicotinyl-2-isopropyl hydrazine phosphate (iproniazid) were dissolved in H_2O and administered orally. Doses of drugs used refer to free base content. Following injection of TA the animals were observed for 60-75 seconds for signs of convulsive activity, then sacrificed by decapitation. Brains were immediately removed, rinsed free of adsorbed blood, blotted lightly, quickly weighed and placed in chilled glass tissue grinder of Potter-Elvehjem type fitted with teflon pestle. For determination of TA the brains were homogenized and analyzed according to method of Hess and Udenfriend(3). Separate brains were homogenized

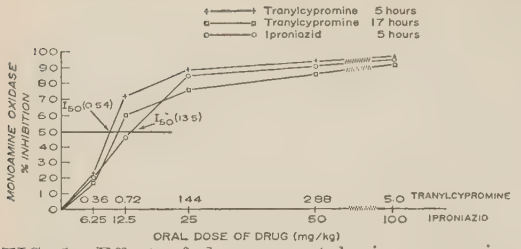

FIG. 1. Effect of drug on rat brain monoamine oxidase activity *in vivo*. Arrows indicate dose (in parentheses) of drug that produced 50% inhibition (I_{50}).

and assayed for MAO activity according to procedure of Bogdanski *et al.*(4).

Results. TA concentration of 14 individual rat brains was 0.032 ± 0.002 μg/g (mean ± S.E.M.). Oral administration of 0.72 mg/kg of tranylcypromine or of 25 mg/kg of iproniazid had no apparent effect upon normal brain TA concentration after 5 or 17 hours.* Sixty seconds after i.v. injection of 5 mg/kg of TA, brain concentration was increased to 0.173 ± 0.019 μg/g (mean of 10 brains ± S.E.M.). Injection of this dose of TA alone did not elicit observable clonic convulsions, although all animals appeared momentarily stunned shortly after injection.

In all rats pretreated with either tranylcypromine or iproniazid clonic convulsive activity was observed 30-40 seconds after injection of TA. With 0.72 mg/kg of tranylcypromine or 25 mg/kg of iproniazid, convulsions persisted for more than 3 seconds and were quite strong. In all animals brain TA concentration was markedly elevated. With 0.36 mg/kg of tranylcypromine the occurrence, duration and relative intensity of clonic convulsive activity appeared dependent upon relative brain TA concentration of the individual animal.

The results (Fig. 1) describe the effect of increasing doses of tranylcypromine and of iproniazid upon rat brain monoamine oxidase activity. Each point is an average of 3 to 6 individual brain values. In addition to showing that tranylcypromine was about 25 times more potent an inhibitor than iproniazid, the curves indicate that 20% inhibition was produced by 0.36 mg/kg of tranylcypromine, while 60-70% inhibition was obtained with administration of 0.72 mg/kg. These results together with data of Table I suggest that occurrence, duration and intensity of TA-induced convulsions were also related to degree of inhibition of monoamine oxidase activity of the rat brain.

Discussion. Hess *et al.*(5) recently suggested that marked central effects, including convulsions, in rabbits and dogs injected i.p. with massive dose (800 mg/kg) of tryptophane after pretreatment with iproniazid were related to measured increase in brain TA concentration. At the same time they considered the possibility that appreciable increases in brain serotonin occurred. More recently Hess *et al.*, in an abstract(6), reported that "characteristic excitement" produced in rats pretreated with monoamine oxidase inhibitor followed by tryptophane or 5-hydroxytryptophane was in no way directly related to brain levels of serotonin and tryptamine. Thus, it was concluded "that increase of brain indoleamines is not in itself sufficient to produce excitement." Our results, however, indicate existence of a good correlation between elevated brain levels of TA and clonic convulsive seizure activity, as described by Tedeschi *et al.* Furthermore, in view of demonstrated ability of systemically injected TA to penetrate the rat brain, it is reasonable to conclude that the elicited pharmacologic effects represented a central mechanism of action of the amine, potentiated by inhibition of brain MAO activity. The question may be raised, however, whether the interaction of the drugs in spinal cord itself may have contributed to production of clonic convulsive seizure activity.

Summary. Normal rat brain tryptamine concentration was 0.032 ± 0.002 μg/g (mean ± S.E.M.). Oral administration of 0.72 mg/kg of tranylcypromine (trans 2-phenylcyclopropylamine) or 25 mg/kg of iproniazid (1-isonicotinyl-2-isopropyl hydrazine) had no apparent effect upon normal rat brain tryptamine concentration. Sixty to 75 seconds after i.v. injection of non-convulsive dose of tryptamine (5 mg/kg) brain concentration of the amine was increased to 0.173 ± 0.019 μg/g. Occurrence, duration and relative intensity of clonic convulsions produced by injec-

* Hess *et al.*(5) reported similar results with guinea pigs pretreated with iproniazid.

TABLE I. Effect of Pretreatment with Tranylcypromine or Iproniazid and Subsequent Injection of Tryptamine upon Brain Tryptamine Levels and Convulsive Activity.

	Iproniazid		Tranylcypromine			
Dose (mg/kg)	25		0.36		0.72	
Pretreatment time (hr)	5	17	5	17	5	17
	Tryptamine conc., μg/g brain (clonic convulsions)*					
	1.080 (+3)	.902 (+3)	.218 (1)	.411 (+3)	1.30 (+3)	.504 (+3)
	.460 (+3)	.779 (+3)	.200 (0)	.175 (0)	.938 (+3)	.451 (+3)
	.648 (+3)	.888 (+3)	.145 (0)	.350 (1)	.699 (+3)	.348 (+3)
	.845 (+3)	.478 (+3)	.500 (+3)	.217 (0)	.856 (+3)	
	.669 (+3)	.605 (+3)	.164 (1)	.388 (+3)	.612 (+3)	
			.059 (0)	.278 (2)	.936 (+3)	
			.195 (1)		.768 (+3)	
					.369 (+3)	
					.252 (+3)	

Dose refers to oral administration of tranylcypromine and iproniazid at indicated time prior to intrav. inj. of tryptamine (5 mg/kg).

* No. in parentheses represents duration of clonic convulsions, in sec. In all cases of (+3), clonic convulsions were sustained beyond 3 sec. and, in most cases, were very marked. Convulsions were elicited 30-40 sec. after tryptamine inj. and animals were sacrificed 60-75 sec. after inj. Control values of tryptamine concentration (μg/g ± S.E.M.):

Normal, .032 ± .002 (N = 14); after either drug (N = 10), same as normal; after tryptamine, .173 ± .019 (N = 10).

tion of tryptamine into rats pretreated with either of above monoamine oxidase inhibitors were correlated with increased concentration of amine in the rat brain. In view of demonstrated ability of systemically injected tryptamine to penetrate rat brain, it is reasonable to conclude that the elicited pharmacologic effects reflected a central mechanism of action of the amine, potentiated by inhibition of brain monoamine oxidase activity.

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Properdin Titers of Dogs Surviving Hemorrhagic Hypotension.* (25763)

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Attention has been called to a relationship between serum properdin levels and hemorrhagic shock(1). It was demonstrated that properdin titers, measured by the zymosan assay, dropped progressively during oligemic and post-transfusion periods of shock. More recently, by phage neutralization assay, titers decreased after hypotensive period had been terminated(2). In these studies, duration of hemorrhage was quite prolonged, and all animals died. To explore further the relationship between properdin and hemorrhagic shock, duration and intensity of hemorrhage was utilized so that approximately 50% of dogs survived. A second group of dogs was subjected to more severe conditions of hypotension so that irreversibility might be expected in virtually all cases. These animals,

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TABLE I. Comparison of Data on Surviving and Non-surviving Dogs.

	No. of dogs	Max bleeding vol, ml/kg	% take up	Survival, hr	Ph.N_{50}/ml properdin titer Control	% control at 6½ hr
Survivors	7	51.3 ± 12.9*	11.6 ± 9.5	†	27.6 ± 8.5	83.7 ± 10
Non-survivors	8	49.7 ± 8.5	18.7 ± 9.9	14.9 ± 3.4	23.0 ± 7.	62.7 ± 13.6
P value		.8	.2		.3	<.01

* Mean ± stand. dev.

† Lived beyond 3 days.

however, were treated by perfusion of intestine through the superior mesenteric artery. Some dogs survived, and properdin studies are presented here.

Methods. Fifteen dogs, weighing 11 to 18 kg, were deprived of food but not water, for 24 hours. They were given 30 mg morphine sulfate intramuscularly one hour before experiment. Procaine was injected locally, and using aseptic technic, both femoral arteries and femoral vein were cannulated. Heparin,† 2 mg/kg i.v. was administered, and additional 5-10 mg given at end of first hour of oligemia. One artery was connected to mercury manometer and the other to sterile graduated reservoir. The animal bled into the reservoir until its arterial pressure fell to 35 mm Hg. Blood pressure was maintained at this level for 135 minutes, by automatic device(3). At end of oligemic period, the blood remaining in reservoir was returned to the dog. Twenty ml samples of blood were taken 5 min. before oligemia, 3-5 min after return of reservoir blood and 6½ hours after start of hemorrhage. Additional bleedings were taken from surviving animals at approximately daily intervals. Blood samples were centrifuged in the cold. Plasma was placed in sealed tubes and stored at –30°C. The phage neutralization method was used to determine properdin titers(4). Activity is expressed as 50% neutralizing units/ml of undiluted plasma (Ph.N_{50}).‡ Hemodynamic data from second group of dogs has been described(5). These animals were subjected to a severity of hemorrhage which ordinarily produces irreversibility. They were kept at 35 mm Hg arterial pressure until they took back 40% of their maximum shed blood volume, or until they had been in oligemia 4-5 hours. In addition, their intestinal vascular beds were perfused with arterial blood at approximately normal rate of flow. Of 7 surviving dogs, 2 had been autoperfused and 5 were donor cross perfused. Many more of perfused dogs died than survived. Data from 6 representative non-survivors are included in this report.

Results. Table I includes data from the 15 dogs subjected to 135 minutes of hypotension at 35 mm Hg arterial pressure. Comparison of results indicates that the only significant difference ($P<0.01$) is that properdin titers 6½ hours after start of hemorrhage decreased to a greater extent in non-survivors.

Fig. 1 records percent change in properdin titer for each dog in terms of its control titer. At the end of hypotensive period (2¼ hr), there was no significant difference between survivors (open circles) and non-survivors (solid triangles). At 6½ hr, however, the percentage decline in properdin titers fell to considerably greater extent in animals which did not survive. The 2 non-survivors which lived 24 and 36 hours showed continued decline in properdin titer (dashed lines). On the other hand, titers of survivors remained at approximately the 6½ hour level until 24 hours had elapsed. Between 24 and 48 hours, level in surviving animals increased to approximately control range. They reached a peak, which in most cases was well above control level, between 4th and 8th day, then started to decline toward control.

Fig. 2 represents properdin levels of 2 autoperfused and 5 donor cross-perfused survivors together with 6 similarly treated non-survivors. Non-survivors had lower properdin titers in immediate post-transfusion period than surviving group. Levels in survivors usually rose to above control within a few

† Heparin supplied thru courtesy of Lilly Labs.

‡ The authors express appreciation to Dr. James Barlow for providing T2r+ phage.

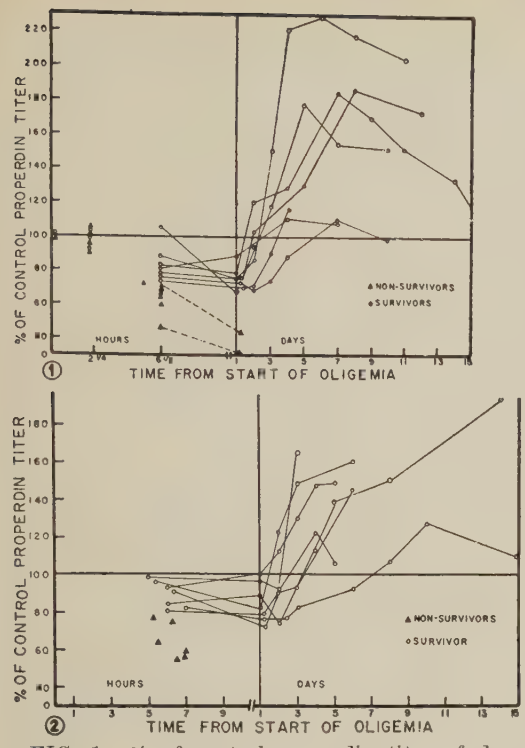

FIG. 1. % of control properdin titer of dogs subjected to hemorrhagic shock at 35 mm Hg arterial pressure for 135 min.

FIG. 2. % of control properdin titer of perfused dogs subjected to severe conditions of hemorrhagic shock.

days and followed the general pattern of survivors in Fig. 1. In perfused dogs, control properdin level was 25.1 ± 12.3 $Ph.N_{50}$ units/ml for survivors and 22.5 ± 9.1 for non-survivors. Duration of oligemia was the same for both groups, 3.8 ± 1 hours.

Discussion. During 135 minutes of oligemia, there was no detectable alteration in serum properdin titer (Fig. 1). The titer declined appreciably, however, 255 minutes after reinfusion of shed blood. This pattern was identical with that reported previously(2) using the same assay.

Initial fall and subsequent rise of properdin titers supplement one of the predominant hypotheses currently invoked to explain hemorrhagic shock. Convincing evidence has been adduced which demonstrated that during oligemia there is a rise in endotoxin content of blood serum(6), and these endotoxins are in some way responsible for resultant lethal outcome(7). The endotoxins are polysaccharides and are probably largely derived from the Gram negative bacteria of intestinal flora(7). Furthermore, bacterial endotoxins are capable of combining with properdin and quantitatively reduced its activity(8).

It may be postulated that endotoxins are formed and combine with properdin to produce reduction in titer during post-transfusion phase. The level of properdin may thus be an inverse measure of amount of endotoxin present. It may be observed that 6½ hours after inception of hemorrhage (Fig. 1) dogs having lowest properdin titer (most endotoxin?) did not survive.

The subsequent rise in properdin level in surviving animals may be due to the stimulating (perhaps antigenic) effect of abnormal amounts of endotoxin resulting from oligemia. Injections of polysaccharides(9) as well as bacterial endotoxin(10) give rise to increased properdin titers. A similar relationship between zymosan and bactericidal activity was previously reported(9) and it was suggested that the polysaccharide stimulated production of antibodies with receptor configurations similar to those found in properdin of normal serum.

Summary. Fifteen dogs were bled to mean arterial pressure of 35 mm Hg for 135 min, after which shed blood was reinfused. Seven animals survived longer than 72 hours. Serum properdin levels fell in all dogs during post-transfusion phase but titers of non-survivors were significantly more depressed than those of survivors. In survivors, properdin levels rose during next few days and reached a level considerably above control by 4th to 8th day, then started to decline toward control. Dogs subjected to more severe conditions of hypotensive shock but perfused by way of superior mesenteric artery showed a similar pattern.

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Reduced Caries in Offspring of Rats Receiving Tetracycline* During Various Prenatal and Post-Partum Periods. (25764)

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It has been shown that aureomycin (chlortetracycline) inhibits caries in the rat when added to a caries-test diet after weaning(1,2). The present communication demonstrates the effectiveness of tetracycline in markedly reducing caries activity in offspring of mothers receiving the antibiotic during various prenatal and post-partum periods.

Methods. Sprague-Dawley rats were bred and insemination was diagnosed by vaginal smear. The animals were then divided into 4 groups. Groups A, B, and C received a solution containing 2.5 mg of tetracycline hydrochloride† per ml prepared fresh 3 times weekly, and Group D received distilled water. Group A received the antibiotic during gestation only (21 days); Group B, during gestation and 14 days of lactation; and Group C, during gestation, lactation and the remaining 7 days of the preweaning period. Group D received distilled water during gestation, lactation and until the offspring were weaned at 21 days of age. Purina laboratory chow was available during gestation and lactation and "non-cariogenic" Diet 550(3) was offered thereafter until the offspring were weaned. The offspring were then housed 2 per cage by sex and received distilled water and caries-test Diet 636(4) *ad libitum* for 60 days. Food and fluid intakes of mothers and offspring were recorded. The heads were cleaned of soft tissue using the dermestid beetle(5), and the teeth were scored for caries(6). The data are presented in Table I. A preliminary investigation which prompted the present extended study was comprised of groups similar to B and D, and the data are presented in Table II.

Results. Tetracycline did not affect fluid consumption of the mothers although the pH of the antibiotic solution was 3.0 (Tables I, II). In addition, tetracycline showed no adverse effect on litter production. Average number of offspring per litter for the 4 groups in Table I was 8.0, 10.6, 9.7 and 8.7 respectively. Because a portion of each litter was reserved for a separate study, only about half of each litter is represented. Tetracycline did not adversely affect weight gains or diet and fluid intakes of the offspring during the 60-day post-weaning experimental period. In the preliminary study (Table II), the control and experimental groups had 7.0 and 9.5 offspring per litter. Weight gains, diet and fluid intakes were somewhat less than in the expanded study. These findings are of interest because Keyes(7) found high mortality in hamsters born of mothers receiving penicillin during gestation and the first week of lactation. Rats appeared to show a better tolerance to penicillin(7).

In the present studies, the most striking effects of administration of tetracycline were

* We are grateful for cooperation of Dr. Stanton Hardy, Lederle Labs., Am. Cyanamid Co., Pearl River, N. Y., in supplying tetracycline hydrochloride.

† Stability of tetracycline hydrochloride as administered to the rat in drinking water was determined spectrophotometrically daily for 3 days. The characteristic spectrum(8) remained unchanged but optical density at the 2 maxima of 276 and 356 mu were depressed 9.7% and 9.4% respectively.

TABLE I. Weight Gain, Diet and Water Intake and Caries Evaluation of Rats Receiving Tetracycline in the Drinking Water during Various Prenatal and Post-partum Periods.

Group		A	B	C	D
Period of tetracycline intake*		G	G + L	G + L + PW	None
Mothers					
Fluid intake†	ml/day	53.1	50.2	49.0	47.3
Tetracycline intake	Total g	1.8	4.0	5.2	.0
Offspring‡					
Litters§	No.	9	11	11	22
Rats	No.	36	46	41	70
Wt gain	g/day	2.4	2.4	2.4	2.1
Diet intake	g/day	12.8	12.9	13.9	12.6
Fluid "	ml/day	17.1	15.4	20.3	15.6
Caries evaluation					
Rats with caries	%	50.0	71.7	48.8	95.7
Carious teeth/rat	No.	1.4	2.9	1.2	7.2
" areas/rat	No.	1.9	4.4	1.7	15.9
Caries score		2.3	4.5	1.8	20.3

* G = During gestation; G + L = During gestation and 14 days of lactation; G + L + PW = During gestation, lactation and the remaining 7 days prior to weaning. Group D consisted of controls and received distilled water for periods G + L + PW.

† Based on 42 days (21 days gestation + 21 days post-partum).

‡ During post weaning period of 60 days.

§ Only about half of the offspring/litter is represented in this study since the remainder was reserved for another study. Mean No. of offspring/litter was 10.6, 9.7, 8.7 and 8.0, respectively.

marked reduction in caries activity under all experimental regimens, and the high degree of fluorescence in the teeth. Mothers receiving the antibiotic during gestation and lactation showed a marked fluorescence of the incisors under ultraviolet light while almost none could be seen in the molars. However, both molars and incisors of the progeny at weaning showed the typical intense golden-yellow fluorescence previously observed in bone(8), thus again indicating that the antibiotic is localized in newly-calcifying tissues.

TABLE II. Weight Gain, Diet and Water Intake and Caries Evaluation of Rats Receiving Tetracycline in the Drinking Water during Gestation and 14 Days of Lactation.

		Control*	Exp.*
Mothers			
Fluid intake†	ml/day	37.3	42.9
Tetracycline intake	Total g	.0	3.9
Offspring‡			
Litters	No.	6	4
Rats	No.	42	38
Wt gain	g/day	1.4	1.5
Diet intake	g/day	9.9	9.8
Fluid "	ml/day	12.5	11.6
Caries evaluation			
Rats with caries	%	100.0	63.2
Carious teeth/rat	No.	9.4	1.7
" areas/rat	No.	30.3	2.7
Caries score/rat		44.7	3.1

* Control group received distilled water during gestation, lactation and 7 days prior to weaning. Experimental group received tetracycline during gestation and 14 days of lactation and then distilled water until weaned at 21 days of age.

† Based on 42 days (21 days gestation + 21 days post-partum).

‡ During post-weaning period of 60 days.

Detailed studies to be presented separately indicate that the extremely low level of caries activity in the tetracycline treated animals is probably not due to presence of the antibiotic in the teeth and lend support to the concept (7) that dental caries is an infectious process transmitted by the intestinal flora. The present studies suggest that tetracycline may be a valuable tool in depressing this flora and thus may provide a baseline for studying the effect of various imposed factors on experimental caries.

Summary. Offspring of mothers receiving tetracycline during various prenatal and post-partum periods showed markedly less caries activity during subsequent 60-day post-weaning assay period. Tetracycline administration did not adversely affect litter production, weight gains, diet intake or fluid consumption in either mother or offspring.

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Stoichiometric Relation between Liver-Receptor, Intrinsic Factor and Vitamin B_{12}.* (25765)

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Since the original report that hog intrinsic factor concentrate (HIFC) enhanced Co^{60}-labeled Vit. B_{12} uptake by rat liver slices(1), the mechanism of this phenomenon has been extensively studied. Data obtained confirm the hypothesis that receptors for intrinsic factor exist on rat liver slices and the low speed precipitate of the homogenate thereof. These receptors can "take up" either free intrinsic factor or intrinsic factor to which Co^{60}-B_{12} is attached(2). Identical receptors for intrinsic factor on small intestinal mucosa of the rat have also been demonstrated(3). The present report extends these studies and confirms the stoichiometry implied previously (2,4).

Materials and methods. Liver homogenate was prepared by mincing fresh (or fresh frozen) liver with scissors, adding 40 ml of 0.9% NaCl per 10 g liver, and homogenizing for 1 minute at room temperature in a Waring blendor. The homogenate was centrifuged at 3000 rpm for 5 minutes, the supernate discarded, and the remaining homogenate washed twice in 0.9% NaCl (40 ml/10 g initial liver tissue). The washed homogenate of "liver debris" (mainly cell walls and nuclei) was suspended in Krebs-Ringer-Tris containing 10 mM $CaCl_2$ (KRT) or in 0.9% NaCl containing 10 mM $CaCl_2$(NaCl-$CaCl_2$); 10 ml of medium was used per 1 g of whole liver starting material. Although it was generally used immediately, washed homogenate prior to suspension in medium may be refrigerated for as long as 2 hours prior to use if necessary. Incubations were performed for one half hour, in air, at room temperature, with constant gentle shaking. Each incubation beaker contained the washed homogenate from 1 g of liver, or 100 mg of lyophilized liver homogenate, suspended in 10 ml of medium, plus 1 ml of the appropriate added agent. Each incubation was followed by centrifugation at 3000 rpm for 5 minutes, discarding the supernatant fluid by decantation, and 3 washes of the homogenate with 10 ml aliquots of NaCl-$CaCl_2$. Each incubation was preceded by suspending the washed homogenate in 10 ml incubation medium. Although subsequent incubations were usually performed immediately, the homogenate may be refrigerated for as long as 1 hour prior to subsequent incubation without loss of activity. One ml of each agent was added to the liver homogenate suspension. Solid materials were made up to 1 ml in 0.9% NaCl. The HIFC preparation used‡ was potent by Schilling-type testing(6) in a daily oral dose of 5 mg. Unless otherwise specified, 25 μg HIFC was used. The amount of Co^{60}-B_{12} added to each sample was 0.01 μg (10^{-8} g) (specific activ-

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TABLE I. Radioactivity Uptake by Homogenate of Frozen Rat, Bull, Cow, and Rabbit Liver after Sequential Incubation in HIFC and Co^{60}-B_{12}.

Homogenate source		Counts/min.
Rat liver stored 7 days at −20°C	Control	14 ± 1
	HIFC	1397 ± 67
Bull liver stored 1 day at −20°C	Control	43 ± 1
	HIFC	1016 ± 40
Cow liver stored 1 day at −20°C	Control	77 ± 8
	HIFC	643 ± 62
Rabbit liver stored 4 days at −20°C	Control	38 ± 8
	HIFC	2184 ±165
Rat liver homogenate precipitate stored 26 days at −20°C	Control	56 ± 7
	HIFC	870 ± 43
Rat liver homogenate suspended in KRT, stored 5 days at −20°C	Control	56 ± 7
	HIFC	145 ± 1

ity = 1 μc/μg). The well type sodium iodide crystal used had a sensitivity of 1 count/min/$\mu\mu$c. *Sequential* incubation (*i.e.*, incubation with HIFC followed by incubation with Co^{60}-B_{12}) was generally performed for reasons stated elsewhere(2,5). After final incubation, retained radioactivity of thrice-washed homogenate was determined, and results recorded as counts per minute per g of initial liver tissue (or per 100 mg of lyophilized liver homogenate). Lyophilized rat liver homogenate was prepared from liver minced with scissors, homogenized in 0.9% NaCl, washed thrice in 0.9% NaCl, and suspended in 0.9% NaCl (or NaCl-$CaCl_2$ if preincubated with HIFC) prior to lyophilization. About 5 g of lyophilized homogenate was derived from 100 g of liver tissue. Acetone-washed lyophilized liver homogenate was prepared by grinding lyophilized homogenate in a mortar with aliquots of acetone until supernate was clear. The final acetone-homogenate mixture was filtered and homogenate remaining on the filter paper was allowed to stand at 4°C until dry. Resultant material was stored at 4°C until used. Rat stomach homogenate was prepared as follows: the frozen stomachs of 10 rats, with the intrinsic factor-free proximal translucent portions discarded(7), were homogenized in 50 ml 0.9% NaCl in a Waring blendor at 6°C for 2 minutes, and filtered with suction (at 6°C) through gauze to remove particles. Fifteen ml 0.9% NaCl were mixed with the particles on the gauze, filtered, and added to the prior filtrate which was then frozen until used. pH of this material was 6.4.

Results. Table I demonstrates that rat, cow, bull, or rabbit liver frozen whole, or rat liver frozen *as the precipitate* of a homogenate, may subsequently be used as a source of receptors.

Rat liver homogenate frozen *while suspended in KRT* appears to lose most of its receptors (Table I). The receptors are protected to a significant degree by incubation with HIFC prior to freezing (Table II).

Lyophilized or lyophilized and acetone-washed rat liver homogenate retains its ability to be used as a source of receptors (Table III). The receptor-intrinsic factor complex may be lyophilized as a unit and retain its ability to "take up" Co^{60}-B_{12} (Table II).

The receptor-intrinsic factor-Co^{60}-B_{12} complex requires calcium for maintenance of its integrity (Tables III, IV). Calcium appears to maintain the receptor-intrinsic factor bond rather than the intrinsic factor-Co^{60}-B_{12} bond (Table IV).

Quantity of HIFC required to "saturate" homogenate receptors from 1 g of fresh rat or frozen cow liver was about 50 μg, as indicated by failure of amounts greater than 50 μg to still further enhance Co^{60}-B_{12} uptake by the homogenate (Table V). Amounts of HIFC much in excess of that required to saturate the receptors actually produced less enhancement than smaller quantities (Table V).

TABLE II. Co^{60}-B_{12} Uptake by Rat Liver Homogenate Incubated with HIFC, then Stored Prior to Addition of Co^{60}-B_{12}.

Conditions of storage	Time stored (days)	Counts/min.
Exp. A:		
Suspended in KRT at −20°C	1	2347 ± 236
Idem	7	2390 ± 0
"	28	2310 ± 54
Exp. B:		
Not stored	0	4746 ± 0
Suspended in KRT at −20°C	3	1663 ± 16
Exp. C:		
Lyophilized; 5°C	7	1708 ± 116
Idem	28	1975 ± 158

TABLE III. HIFC Effect on Co^{60}-B_{12} Uptake by Fresh, Lyophilized, or Lyophilized and Acetone-Washed Rat Liver Homogenate; Similar in KRT or NaCl-$CaCl_2$ Medium.

Medium	Homogenate	Agent added before first incubation	Counts/min.
KRT	Fresh	NaCl	73 ± 4
"	"	HIFC (.5 mg)	6588 ± 45
NaCl-$CaCl_2$	"	NaCl	85 ± 2
"	"	HIFC (")	7058 ± 75
KRT	Lyophilized*	NaCl	28
"	"	HIFC (")	6533
NaCl-$CaCl_2$	"	NaCl	37
"	"	HIFC (")	5973
KRT	Lyophil.-acet.†	HIFC (.25 mg)	2811 ±251

* 100 mg, stored 4 days at 5°C prior to use. † 100 mg, stored 4 days at 5°C after lyophilization and acetone-washing.

The intrinsic factor-receptor complex is destroyed by heating at 100°C for 10 minutes prior to addition of Co^{60}-B_{12}, although HIFC itself retained about 25% activity after such heating (Table VI).

TABLE IV. Calcium Dependence of HIFC Effect.

1st incubation Medium	1st incubation Added agent	2nd incubation Medium	Counts/min.
Exp. A (fresh liver homogenate)			
NaCl	NaCl	NaCl	58 ± 1
	HIFC	"	159 ± 30
NaCl-$CaCl_2$	NaCl	NaCl-$CaCl_2$	102 ± 86
	HIFC	"	3196 ±445
	NaCl	NaCl	44 ± 4
	HIFC	"	70 ± 1
NaCl	NaCl	NaCl-$CaCl_2$	34 ± 4
	HIFC	"	104 ± 37
Exp. B (lyophilized liver homogenate)			
KRT	HIFC	KRT	2049
KRT-Ca*	"	"	19

* Calcium replaced by equivalent millimolar concentration of sodium.

Rat stomach homogenate, pH 6.4, enhanced Co^{60}-B_{12} uptake by rat liver homogenate (Table VI), but its effect was reduced by standing for 30 minutes at pH 2.6. The stomach homogenate after standing 2 hours at 3°C at pH 6.4, retained much, but not all, of its original activity (Table VI).

TABLE V. Effect of Varying HIFC Concentration on Co^{60}-B_{12} Uptake by Fresh Rat and Frozen Cow Liver Homogenate.

Agent added prior to 1st incubation	Rat liver Counts/min.	Cow liver Counts/min.
NaCl	56 ± 7	77 ± 8
25 μg HIFC	1264 ± 171	643 ± 62
50 μg "	3994 ± 14	1084 ± 93
100 μg "	4021 ± 41	973 ± 78
500 μg "	3410 ± 200	211 ± 34

TABLE VI. Depression of HIFC and Rat Stomach Homogenate Effects by Heat or Low pH.

	Agent added prior to 1st incubation	Counts/min.
Exp. A	NaCl	94 ± 5
	HIFC	1835 ± 52
	" *	58 ± 3
	" heated 10 min. at 100°C	517 ± 52
Exp. B	NaCl	33 ± 2
	HIFC	1740 ± 30
	RSH†	865 ± 37
	" †, heated 30 min. at 37°C, pH 2.6	341 ± 6
	" †, after 2 hr at 3°C	659 ± 35

* Prior to 2nd incubation, homogenate heated 10 min. at 100°C.
† Rat stomach homogenate.

Gradual reduction of amount of Co^{60}-B_{12} added prior to the second incubation resulted in a gradual increase in percentage of Co^{60}-B_{12} remaining on the homogenate "receptor-intrinsic factor complex" after incubation (Table VII).

By varying the quantities of HIFC and of Co^{60}-B_{12} within the limits imposed by the quantity of receptors on liver homogenate, a point is approached where a stoichiometric relationship exists between these quantities (Table VIII).

When a mixture of Co^{60}-B_{12} and non-radioactive B_{12} (cold B_{12}) is added to liver homogenate preincubated with HIFC, a reduction of Co^{60}-B_{12} uptake is observed which is proportional to amount of "cold" B_{12} used (Table IX).

Discussion. The present demonstration

TABLE VII. Co^{60}-B_{12} Uptake by Rat Liver Homogenate, Using Sequential Incubation and Decreasing Amounts of Co^{60}-B_{12}.

Co^{60}-B_{12} added prior to 2nd incubation ($\mu\mu g$)	Counts/min.
9660	2308 ±20
4830	1753 ± 3
2415	1205 ± 1
966	720 ± 9
97	92 ± 2

that the "intrinsic factor receptors" on mammalian liver remain intact after lyophilization and acetone extraction of the liver indicate the possibility of further purification of these receptors, with the ultimate goal of a pure system of receptors, intrinsic factor, and B_{12}. If there is a stoichiometric relationship between these 3, as the present data indicate, it becomes possible to assay the amount of any one of these substances by determining the others.

Until intrinsic factor is isolated in pure form, the nature of the material combining with the receptors will remain uncertain. The activity may be due to a contaminant common to HIFC, rat stomach homogenate, and normal human gastric juice(2). Similarly, degraded intrinsic factor or other materials with the appropriate prosthetic group(s) may attach to receptors and block uptake of intrinsic factor(8,9), and intrinsic factor to which "cold" B_{12} is bound cannot be assayed in this system(8). Excessive amounts of intrinsic factor must be avoided to prevent erroneous results due to loose attachment of excess HIFC to the homogenate. Some of this may not be removed in the washes and be released into the incubation medium during subsequent incubation with Co^{60}-B_{12} to compete with intrinsic factor on receptors for available B_{12} (2). Optimum amount of intrinsic factor to use for intrinsic factor assay would be that quantity sufficient to enhance Co^{60}-B_{12} uptake markedly, but not to saturate receptors.

TABLE VIII. Effect of Varying Quantities of HIFC and of Co^{60}-B_{12} in Sequential Incubation System with Rat Liver Homogenate.

HIFC (μg)	Co^{60}-B_{12} ($\mu\mu g$)	Counts/min.
0	2898	14 ± 1
20	2898	888 ± 93
20	1932	746 ± 79
20	966	437 ± 74
25	2898	1208 ± 8
25	1932	1074 ± 13
25	966	591 ± 44
30	2898	1533 ± 82
30	1932	1102 ± 45
30	966	592 ± 6

Apropos of the possible relation of the present system to the physiologic system for absorption of B_{12} from the human small intestine(2), it should be noted that in the human small intestine there is also a stoichiometric relation between intrinsic factor and absorption of B_{12}(10).

TABLE IX. Reduction of Radioactivity Uptake by Rat Liver Homogenate in Sequential Incubation System When Cold B_{12} Mixed with Co^{60}-B_{12}.

Agent(s) added before incubation: 1st incubation	2nd incubation	Counts/min.
Exp. A:		
NaCl	Co^{60}-B_{12} + NaCl	20 ± 1
HIFC	" + "	1117 ± 17
	" + 10,000 $\mu\mu g$ B_{12}	432 ± 6
	" + 5,000	612 ± 19
	" + 2,500	768 ± 1
	" + 1,000	932 ± 16
Exp. B		
NaCl	Co^{60}-B_{12} + NaCl*	27 ± 1
HIFC	" + NaCl*	1097 ± 27
	" + 5,000 $\mu\mu g$ B_{12}*	660 ± 52
	" + 2,500*	771 ± 1
	" + 1,250*	895 ± 8
	" + 500*	966 ± 20
	" + 250*	1019 ± 9
	50*	1028 ± 37

Co^{60}-B_{12} used was one-half ml, specific activity 1 $\mu c/\mu g$; 4,465 $\mu\mu g/½$ ml.

* In 5 ml of 0.9% NaCl.

Rat intrinsic factor (rat stomach homogenate) is capable of marked enhancement of Co^{60}-B_{12} uptake by rat liver homogenate in the *sequential* incubation system here used. This latter point is important in view of the failure of homologous gastric juice to enhance Co^{60}-B_{12} uptake by rat liver slices in a *simultaneous* incubation system(11,2).

Summary. "Receptors" for intrinsic factor exist in rat, bull, and rabbit liver homogenate precipitate, and remain intact after lyophilization and acetone-washing of the homogenate. There appears to be a stoichiometric relation between receptors, intrinsic factor, and Vit. B_{12}. Within stated limits, an incubation system of receptors, intrinsic factor, and/or Co^{60}-B_{12} may prove useful for assay of intrinsic

factor, of the quantity of free B_{12} in biological fluids, and of receptors.

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Removal of Chylomicron Lipid from Plasma in Experimental Nephrosis.* (25766)

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Hyperlipemia is a characteristic feature of experimental nephrosis produced in rats by injection of rabbit anti-rat kidney serum. One factor contributing to the hyperlipemia is a decrease in rate of removal of chylomicron lipid from the circulation(1,2). Two possible explanations for this abnormality are suggested. First, the delayed removal may be dependent on presence in nephrotic plasma of an inhibitor to lipoprotein lipase which is responsible for intravascular lipolysis of chylomicrons(3). Second, the suggestion has been made that nephrotic hyperlipemia is due to retention of lipid in the plasma as the result of hypoalbuminemia, on the assumption that the amount of serum albumin is insufficient to transfer fatty acids at a normal rate from lipoproteins to the tissues(4). The following studies were undertaken to assess the effects of serum albumin concentration and of lipoprotein lipase inhibitor on rate of chylomicron removal from the circulation of rats made nephrotic by antikidney serum.

Methods. Nephrosis was produced in rats by injection of rabbit anti-rat kidney serum (5). Rat serum albumin was obtained from normal rat serum after precipitation of globulins by third-saturated ammonium sulfate. It was dialyzed against normal saline to remove excess sulfate, lyophilized, and made up in concentrated solution for administration. On electrophoretic examination the albumin was 87% pure. For *in vitro* studies on clearing of lipemic serum, the source of lipoprotein lipase was post-heparin plasma, obtained from the aorta of fasted normal rats 15 minutes after intravenous injection of heparin (4 mg/kg body weight). Post-heparin plasma was incubated with normal rat serum previously made lipemic by addition of rat chyle sufficient to give an optical density (O.D.) of 0.450-0.700 when read at 550 mμ or 750 mμ. In a preliminary experiment the O. D. of lipemic serum was found to be a linear function of amount of rat chyle added to it. To measure rate of removal of chylomicrons from the circulation, 1.0 ml of C^{14}-labeled chyle, prepared according to French and Morris(6), was injected intravenously; radioactivity was measured in whole blood at intervals over the next 30 minutes, by plating aliquots of 0.05 ml of tail vein blood and counting in a windowless proportional gas flow counter. The fractional rate constant of chylomicron re-

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moval (fraction/min.) was then taken as the slope of the log of radioactivity (counts/min/ml) plotted against time. Freshly prepared chyle was used for each experiment; lipid concentrations varied from one preparation to another, but in each experiment all animals received the same amount of lipid. Chylomicron counts were made on serum in a standard field under dark ground illumination. Animals were fed a standard laboratory chow and given tap water to drink, unless otherwise noted.

Results. Effect of glucose feeding on chylomicronemia. It was previously reported(2) that withdrawal of food and giving of 50% glucose to drink for 16 hours resulted in low levels of endogenous chylomicronemia in both normal and nephrotic rats. To confirm this finding, chylomicron counts were made on 4 normal and 4 nephrotic rats prepared in this way. In addition, 1 normal animal so prepared was given 1.0 ml of chyle intravenously, and serum was taken at once for a chylomicron count. Counts in the standard field were: normal, 3-5; nephrotic, 5-10; normal chyle-injected, 550. Thus the levels of endogenous chylomicronemia were insignificant in the presence of the level produced by injection of chyle; consequently in all subsequent experiments based on measurement of chylomicron removal rates, animals were prepared in this manner to minimize dilution of exogenous with endogenous chylomicrons.

Effect of endogenous hyperlipemia on rate of disappearance of chylomicrons from blood. Two rats were fed a diet containing 60% butter-fat and 5% cholesterol; this produced fasting serum lipid levels of 5.4 and 4.9 g/100 ml. Rate of removal of chylomicrons from the circulation was then measured in these animals and in 2 control animals given the standard diet. The fractional rate constants of removal were essentially the same for the 2 pairs of rats: 0.038 and 0.053 for control animals and 0.042 and 0.043 for hyperlipemic animals.

Effect of albumin administration on rate of disappearance of chylomicrons from blood of nephrotic rats. Six nephrotic rats were used, each serving as its own control. Labeled chyle was injected into each rat intravenously twice at an interval of 2-3 days, and disappearance rate of radioactivity from the blood was measured. Ten minutes before the second injection, each rat received 1 ml of rat serum albumin (9.0 g/100 ml), sufficient to increase serum albumin by approximately 1.5 g/100 ml. Fractional rate constants before and after albumin administration are listed in Table I. Results indicate that except in one rat (#6) raising the levels of albumin failed to raise fractional rate constant of removal to a significant degree.

TABLE I. Effect of Albumin on Fractional Rate Constant of Chylomicron Disappearance in Nephrotic Rats.

	Fraction/min.			Fraction/min.	
No.	Control	Post albumin inj.	No.	Control	Post albumin inj.
1	.0014	.0037	4	.0003	.0011
2	.0082	.0113	5	.0018	.0056
3	.0022	.0027	6	.0070	.0554

Susceptibility of chylomicrons from normal and nephrotic rats to post-heparin clearing factor in vitro. To aliquots of normal rat serum was added chyle collected from normal or nephrotic rats to give "lipemic sera" of equal O.D. To 0.9 ml aliquots of these sera was added 0.1 ml of normal post-heparin plasma, and mixtures were incubated at 35°C for 90 minutes, with readings of O.D. made every 30 minutes. The results (Table II) fail to show any differences in rates of clearing of chylomicrons obtained from normal and nephrotic rats.

Effect of nephrotic plasma on post-heparin clearing factor in vitro. To determine the effect of nephrotic plasma on clearing rate of lipemic serum by post-heparin plasma, aliquots of 0.1 ml or 0.3 ml of nephrotic and control sera were added to 1 ml of lipemic rat serum before addition of 0.1 ml of post-heparin plasma. The O.D. was read at 0, 30, 60 and 120 minutes. The results (Table III)

TABLE II. Clearance of Normal and Nephrotic Chylomicrons by Post-Heparin Plasma *In Vitro.*

	30 min.	60 min.	90 min.
Normal	.106	.165	.170
Nephrotic	.099	.164	.191

Values represent mean decreases in O.D. in 3 experiments.

TABLE III. Effect of Normal and Nephrotic Sera on Lipemia Clearing *In Vitro*.

Exp.			Conc. of test serum, % of total vol	% change in O.D. 30′	% difference	60′	% difference	120′	% difference
1	Control	(6)	10			27.0	−18.5	34.4	−18.6
	Nephrotic	(6)	10			22.0		28.0	
2	Control	(4)	10			45.3	−30.0		
	Nephrotic	(6)	10			31.7			
3	Control	(6)	10	17.2	−27.9	22.3	−24.7	19.5	−24.1
	Nephrotic	(5)	10	12.4		16.8		14.8	
	Control	(6)	25	16.4	−40.8	22.8	−31.1	26.0	−53.1
	Nephrotic	(5)	25	9.7		15.7		12.2	
4	Control	(6)	10	10.7	−50.5	18.2	−37.9	26.2	−33.6
	Nephrotic	(8)	10	5.3		11.3		17.4	
	Control	(6)	25	6.0	−66.6	12.2	−62.3	19.8	−54.0
	Nephrotic	(8)	25	2.0		4.6		9.1	

Concentration of 10% of test serum obtained by adding 0.1 ml of serum to 0.9 ml of lipemic plasma.

Concentration of 25% by addition of 0.3 ml of test serum to 0.9 ml of lipemic plasma.

indicate that nephrotic plasma in a concentration of 10% by volume caused a 30-50% inhibition of lipemia clearing. An increase in concentration of nephrotic plasma to 25% by volume caused an inhibition of somewhat greater magnitude.

Effect of nephrotic plasma on disappearance rate of chylomicrons from blood of normal rats. Three normal rats were injected with .5 ml of labeled chyle and disappearance rate of radioactivity in blood was measured. Three days later 1 ml of nephrotic serum was given to the same animals, followed in 10 minutes by a second injection of labeled chyle; radioactivity in blood was again measured. The results (Table IV) show that no inhibition of removal occurred in any of the animals as a result of administration of nephrotic serum.

Discussion. Deficiency of serum albumin in nephrotic rats has been suggested as the cause of nephrotic hyperlipemia on the assumption that the available albumin cannot remove the freed fatty acids which then inhibit further lipolysis(4). In support of this hypothesis, it was shown that continuous intravenous infusion of bovine serum albumin lowered the concentration of lipids in the blood of nephrotic rats to nearly normal values(7). However, Morris and French(1) found that administration of 200 mg (equal to the total serum albumin of a normal rat weighing 120 g) of bovine serum albumin produced no acceleration in rate of disappearance of chylomicrons from the blood of normal or nephrotic rats. The present work was based on use of homologous serum albumin in preference to bovine material in order to evaluate the role of albumin under more physiologic conditions. The results, showing failure of an increase in serum albumin concentration to increase rate of removal of chylomicrons, confirm the findings of Morris and French(1), and lead to the conclusion that hypoalbuminemia is not the cause of retention of chylomicron fat in the blood in nephrosis.

TABLE IV. Effect of Nephrotic Plasma on Fractional Rate Constant of Chylomicron Removal in 3 Animals.

(Fraction/min.)

Control	After inj. of nephrotic plasma
.158	.163
.193	.177
.190	.188

It has been postulated that the delayed removal of chylomicrons is the result of isotope dilution by endogenous plasma lipids released from fat depots(1). This thesis makes the assumptions that the endogenous lipemia is due to increased mobilization and that the serum lipids are present in the form of chylomicrons. Our results indicate that the dietary preparation used caused a fall in chylomicronemia to very low levels in both normal and nephrotic rats; though the level was slightly

higher in nephrotic animals, the difference was insignificant in the presence of the marked rise in chylomicronemia produced by injection of the test load of chyle. Furthermore, the presence of massive endogenous hyperlipemia in non-nephrotic rats did not alter rate of removal of administered chylomicrons. It is therefore concluded that the decreased rate of chyle removal from nephrotic plasma was not due to isotope dilution.

An inhibitor of lipoprotein lipase has been demonstrated in the serum of nephrotic rats (3), and the possibility was thus raised that the decreased rate of removal of chylomicrons is a consequence of this inhibition. Our results confirm the observation that nephrotic plasma inhibits the clearing action *in vitro* of post-heparin plasma. However, when normal rats were injected with nephrotic serum to obtain a concentration in their vascular system comparable to that used in the *in vitro* system, no inhibitory effect on rate of chyle removal was observed. This suggests that the clearing process may not play a major role in chylomicron removal. There is evidence that chylomicrons can be removed as particulate matter without prior hydrolysis by serum lipoprotein lipase(8), so that even if the degree of inhibition of lipoprotein lipase observed *in vitro* had obtained *in vivo,* it might not have been apparent in overall estimates of the chylomicron removal rate.

Summary. 1. Carbohydrate feeding reduced chylomicronemia to similarly low levels in normal and nephrotic rats; following such dietary preparation the decreased rate of chylomicron removal from blood of nephrotic rats is not due to dilution of administered chylomicrons. 2. Nephrotic rats were given intravenous injections of 90 mg of normal rat serum albumin. This did not alter rate of removal of labeled chylomicrons from the circulation. 3. When nephrotic rat plasma was added to *in vitro* clearing system, consisting of normal rat plasma, normal rat chylomicrons and normal rat post-heparin plasma, it significantly decreased rate of clearing. Chylomicrons obtained from normal and nephrotic rats were cleared equally well by serum lipoprotein lipase. 4. Injection of nephrotic rat plasma into normal rats did not alter rate of removal of labeled chylomicrons.

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Rapid Adsorption of Vaccinia Virus on Tissue Culture Cells by Centrifugal Force.* (25767)

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Because of their large size, vaccinia virus particles diffuse slowly. The several hours generally allotted to adsorption of a virus inoculum to cells in monolayer can be reduced if adsorption takes place in suspensions in which the cells are quite concentrated(1). However, difficulty is still encountered in attaining adsorption of a high percentage of the virus in a short time. If accurate measurements of the time between adsorption of a

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virus particle to a cell and some subsequent result are to be made it is necessary to know the moment of adsorption rather than only that it must have occurred at some time during a several hour period. In this paper a method employing centrifugal force is described, which brings cells and virus quickly together in a monolayer.

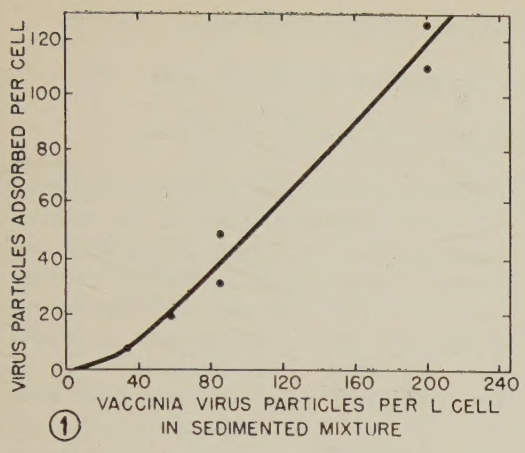
Materials and methods. Vaccinia virus, strain WR, from American Type Culture Collection, adapted to growth in L cells(1) was used in this work. Cells containing 2000 to 3000 virus particles each were stored at −60°C. From this pool, starting virus for the experiments was obtained by breaking the cells with sonic waves(1) to produce suspensions of about 10^9 particles per ml. L cells were grown in a medium containing Earle's balanced salt solution, horse serum and yeast extract(1). Suspensions were made by scraping cells from the glass surface and agitating them by rapid pipetting. Cell counts were made in a hemacytometer. Rapid adsorption of virus to cells is obtained by sedimentation of a suspension of the two in a horizontal tube centrifuge rotor. It is necessary that cells and virus sediment vertically upon a smooth flat surface. This can be produced in the bottom of an ordinary test tube by pouring in about 1 ml of warm melted agar and allowing it to cool. To insure complete freedom from curvature due to meniscus effects at the edges, this cooling is done with the tubes spinning in the centrifuge. The centrifuge rotor must be the horizontal (swinging bucket) type to minimize deposit of material on the walls of tubes. The horizontal, Field-Aligning rotor manufactured by Ivan Sorvall, Inc. of Norwalk, Conn., was used. It has the added advantage, for this work, of double pivots in tube mountings. Tubes swing from vertical to horizontal positions as speed is increased; they swing also somewhat behind or in advance of the radial direction during acceleration and deceleration respectively. This feature tends to assure maintenance of strict perpendicularity between collecting surface and sedimenting forces at all times. The mixture of cells and virus was made to contain just enough cells to form a uniform monolayer on the agar surface. The slower sedimenting virus in the mixture will then fall upon this monolayer where it can be held for any desired time. Following sedimentation the monolayer of cells was removed by rapid pipetting (over 90% were recovered) with growth fluid.‡ The centrifuge tubes were cellulose acetate, 16 mm diameter, and 1.0 x 10^6 cells were used in each tube. Volume of the suspension was such that its depth, over the agar receiving surface, was ½ cm. After sedimentation, cells were washed by suspending them in 5 ml growth medium, sedimenting in pointed tubes, resuspending in a like volume and sedimenting again. Virus was released by exposing the washed cell suspension to 9 kc sonic waves for 2 minutes (1). Virus particles were counted by the sedimentation procedure(1,2) employing electron microscopy and these will be called adsorbed virus.

Results. A series of mixtures of L cells and stock virus was made in 1 ml quantities of growth medium. Each contained 1.0 x 10^6 cells/ml and virus particles in the following concentrations: 34, 58, 85, and 202 x 10^6/ml. These were placed in the swinging bucket centrifuge tubes and the rotor brought up to 8000 rpm (about 8000 x G) in such a manner that increase of speed/unit time was constant. Acceleration time was 1½ minutes. After resuspension and washing of cells, which took an average of approximately 6 minutes, the adsorbed virus was released and counted as described in materials and methods. The results are shown by the graph (Fig. 1) of adsorbed virus particles plotted as a function of virus concentrations in the original mixture.

Preliminary experiments have shown that some of this attached virus is easily eluted from the cells and that new virus first appears, when the multiplicity is high, in about 6 hours from time of inoculation. The cells have shown no evidence of reduction in virus producing ability because of their exposure to the high centrifugal force.

Discussion. When centrifugal force is used to bring cells and virus in contact, the number of virus particles adhering to each cell, after the washing process, is very high. To a

‡ PBS is unsatisfactory for this purpose because with it, cells tend to stick to glass surface.



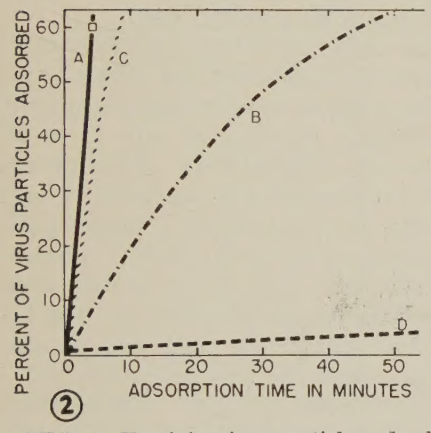


FIG. 1. Vaccinia virus particles adsorbed on L cells by sedimentation from suspensions of various virus concentrations. No. of cells was constant.

FIG. 2. Adsorption of vaccinia virus to L cells when aided by centrifugal force (A) compared with calculated ideal rate for collisions in same suspension (B), ideal rate for a suspension of 5 times greater cell concentration (C), and observed rate for the latter (D).

first approximation, the relationship between particles adsorbed and concentration of the inoculum is linear (Fig. 1). There is no evidence of saturation of cells with virus. There is, however, a noticeable tendency for the line to approach zero in a curve. This tendency is quantitatively the same as that observed in similar experiments using conventional inoculation methods(1). The centrifugal method was designed to bring cells and virus quickly into close proximity so that measurements of the time interval before significant events in infection and growth sequence could be more exact. This it does well as can be seen from the chart, Fig. 2. Calculation based upon the known sedimentation properties of vaccinia virus yields 4½ minutes as the time required for all particles to reach the cell layer. At a multiplicity of 202 (Fig. 1) 60% of the inoculum was adsorbed by the cells (curve A, Fig. 2). If this mixture had been allowed to stand and each collision between virus and cell had resulted in adsorption the calculated adsorption process would have followed curve B.§ This control was not made but one using 5 times as many cells per ml has shown(1) rate of adsorption depicted in curve D. Calculated theoretical maximum for this system would be the rate shown in curve C. Attachment of virus to cells is thus somewhat over 100 times faster than that observed even in suspensions of high cell concentration with a multiplicity of 202.

Summary. By sedimenting a mixture of L cells and vaccinia virus upon a flat surface, 60% adsorption is attained in 4½ minutes. These data have been obtained by destruction of washed cells by sonic waves and counting released virus by electron microscopy.

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§ Calculations based on general equations of Allison and Valentine(3).

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Effect of Long Chain Keto Acids on Encephalomalacia in Chicks.* (25768)

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The presence of unsaturated fatty acids and absence of Vit. E have served as dietary prerequisites to development of a high incidence of encephalomalacia in chicks(1-4). However, as synthetic antioxidants have been shown to decrease the rate of incidence(5-7), exposure of usaturated dietary fats to autoxidation may also represent a factor in the rate of incidence. In the present study 3 possible oxidation products of long chain unsaturated fatty acids were therefore fed to chicks kept on a Vit. E deficient diet and the effect of oxidation products on rate of incidence determined.

Methods. The long chain oxidation products or keto acids (Table I) were prepared from castor oil(8). The 12-oxo-octadecanoic acid was prepared from 12-oxo-*cis*-9-octadecenoic acid by hydrogenation under 50 lb pressure in the presence of palladium-aluminum oxide catalyst. The constants of all the synthesized keto acids agreed with those previously reported(8). Five groups of week-old male chicks (New Hampshire Columbia cross) were fed *ad lib* a Vit. E-deficient diet† and 0.25% of the keto acid. When clinical signs of encephalomalacia(9) such as ataxia, head retraction, and spasms followed by complete prostration were observed in the chick, it was removed from the cage for further histological and chemical determinations. Onset of encephalomalacia in the chick was confirmed by observing gross lesions, accompanied by hemorrhage and edema of the cerebellum. In another study with 12 groups of 6 birds each, levels of both keto (12-oxo-*cis*-9-octadecenoic) acid and corn oil were varied at the expense of glucose on a weight basis.

Results. Addition of long chain keto acids to a typical Vit. E-deficient diet (Table I) induced symptoms of encephalomalacia after 7 days of feeding as compared with 15 to 28 days previously reported(1-4). When the experiment was terminated at the end of 11 days on this diet, all chicks on diet 2 showed positive symptoms of encephalomalacia. 12-Oxo-*cis*-9-octadecenoic acid or its methyl ester caused the highest rate of incidence. The 12-oxo-*trans*-10-octadecenoic acid and 12-oxo-octadecanoic acid were not as potent as 12-oxo-*cis*-9-octadecenoic acid. Furthermore, rate of incidence depended on level of dietary fat as well as level of keto acid. A higher rate of incidence was noted at a 10 than at a 5 or 2.5% level of corn oil (Table II). A level of keto acid as low as 0.05% increased rate of incidence.

Discussion. The presence of oxidation products of long chain unsaturated fatty acids in a diet which contained unsaturated fat was shown to accelerate development of encephalomalacia in chicks. The presence of a dietary source of Vit. E, namely corn oil, which contained a reported level of 100 mg % of mixed tocopherols(10), did not counteract the effect of keto acids. The influence of keto acids on metabolism of Vit. E *in vivo* will be discussed in a subsequent paper.

Summary. The presence of oxidation products of long chain unsaturated fatty or "keto" acids, accelerated development of encephalomalacia in chicks kept on a diet containing corn oil. 12-Oxo-*cis*-9-octadecenoic acid was

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† Each 100 g of basal ration contained 68.18 g glucose, 35.3 g isolated soybean protein, 0.75 g DL-methionine, 0.3 g glycine, 0.2 g choline chloride, 2.166 g calcium carbonate, 1.05 g potassium phosphate (monobasic), 0.940 g calcium phosphate (dibasic), 0.80 g sodium chloride, 0.25 g magnesium sulfate, 0.03 g ferrous sulfate, 0.02 g manganous sulfate, 0.01 g zinc carbonate, 0.002 g cupric sulfate, 0.001 g potassium iodide, 0.001 g sodium molybdate, 10 mg thiamine hydrochloride, 10 mg niacin, 1.6 mg riboflavin, 2.0 mg calcium pantothenate, 0.002 mg vit. B_{12}, 0.6 mg pyridoxine hydrochloride, 0.006 mg biotin, 0.4 mg folic acid, 0.2 mg p-aminobenzoic acid, 0.5 mg menadione, 1000 I.U. vit. A acetate and 60 I.U. vit. D_3. The keto acid was mixed with the basal ration at the beginning of the experimental period and the diets kept in metal cans at room temperature.

TABLE I. Effect of Keto Acids on Severity of Encephalomalacia.

Diet	Supplements to basal diet*	Feed intake, 7 days (g)	Wt gain, 7 days (g)	Positive symptoms
1	10% corn oil	99	100	2/12†
2	Diet 1 + 0.25% 12-oxo-*cis*-9-octadecenoic acid	98	98	12/12
3	Diet 1 + 0.25% 12-oxo-octadecanoic acid	100	105	5/12
4	Diet 1 + 0.25% 12-oxo-*trans*-10-octadecenoic acid	85	96	5/12
5	Diet 1 + 0.25% methyl ester of 12-oxo-*cis*-9-octadecenoic acid	99	96	10/11

* Supplements to basal diet were made at expense of glucose on weight basis.
† Results expressed as fraction with No. of chicks showing advanced stages of encephalomalacia in numerator and total No. of chicks in denominator.

TABLE II. Effect of 12-Oxo-*cis*-9-octadecenoic Acid and Corn Oil Levels on Severity of Encephalomalacia.

Treatment No.	Test diet Supplements to basal % keto acid	% corn oil	Feed intake, 7 days (g)	Wt gain, 7 days (g)	Positive symptoms
1	0	2.5	129	95	0/6
2	0	5.0	127	94	1/6
3	0	10.0	126	107	"
4	.05	2.5	123	97	0/6
5	.05	5.0	124	100	3/6
6	.05	10.0	117	94	"
7	.1	2.5	127	98	1/6
8	.1	5.0	128	100	2/6
9	.1	10.0	116	100	4/6
10	.25	2.5	124	92	1/6
11	.25	5.0	117	93	2/6
12	.25	10.0	118	92	6/6

most potent keto acid tested to date and was most effective in presence of dietary source of unsaturated fatty acid such as corn oil. With basal diet containing 10% corn oil, clinical symptoms of encephalomalacia could be induced at end of 7 days by supplementing the diet with 0.25% of 12-oxo-*cis*-9-octadecenoic acid.

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